Organization of Ribosomal RNA Genes in Thermoplasma acidophilum

(Die Organisation der ribosomalen RNA-Gene in Thermoplasma acidophilum)

Jenn Tu

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Organization of Ribosomal RNA Genes in *Thermoplasma acidophilum*

(Die Organisation der ribosomalen RNA-Gene in *Thermoplasma acidophilum*)

Inauguraldissertation
zur Erlangung des Doktorgrades
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Jenn Tu
aus Kweichow

München 1982

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TO MY PARENTS

The present work was completed in the Abteilung molekulare Biologie der Genwirkung (Department Molecular Biology of Gene Expression) at the Max-Planck Institut für Biochemie, D-8033 Martinsried.

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1. SUMMARIES

1.1 SUMMARY (IN ENGLISH)

RNA species (mainly the rRNAs) and the S-values of Thermoplasma ribosomes have been analysed. The rDNA organization has been carefully worked out both by hybridizing the purified ^{32}P-labeled rRNAs to restriction-enzyme digested Thermoplasma DNA and by rDNA cloning. It has been clearly shown that Thermoplasma has a very special rDNA organization which has never been reported for any other organism.

Ribosomes of Thermoplasma have an S-value of 64S and consist of two subunits, namely the 45S and the 32S nucleoprotein complexes. From the 45S subunit 5S rRNA and two 23S rRNAs were isolated. One of the two 23S rRNAs which has a higher mobility than the other in gel electrophoresis might be an artifact. From the 32S subunit the 16S rRNA, its two precursors and some mRNAs were isolated. The only rRNA species which exist in mature ribosomes are the 5S, 16S rRNAs and the two 23S rRNAs.

There is only one copy of 23S rRNA gene in the Thermoplasma genome; the same probably applies to the 16S and 5S rRNA genes. In contrast to the eubacterial 5'-16S-23S-5S 3' and the eukaryotic 5'-18S-5.8S-28S-3' rDNA

organizations (the latter with independent 5S-DNAs), it seems that the three Thermoplasma rRNA genes are independent from one another and distributed in the genome. The distances between 16S and 23S, 23S and 5S, and 5S and 16S rRNA genes are at least 7.5 Kb, 6 Kb and 1.5 Kb respectively. No linkages between these rRNA genes have been found by analysing the plasmids carrying rDNA or by hybridizing the nick-translated plasmids to restricted Thermoplasma DNA.

1.2 ZUSAMMENFASSUNG (SUMMARY IN GERMAN)

Thermoplasma RNA (insbesondere rRNA) und die Sedimentationskonstanten der Ribosomen und ribosomalen Untereinheiten wurden untersucht. Die Organisation der Gene, die für ribosomale RNA codieren, wurde durch Hybridisierung mit gereinigter rRNA gegen mit Restriktionsendonucleasen fragmentierter Thermoplasma DNA und durch molekulares Klonieren der rDNA, analysiert. Es wurde eindeutig gezeigt, daß in Thermoplasma die rRNA-Gene in bisher nicht beschriebener Weise organisiert sind.

Das Thermoplasma Ribosom besitzt eine Svedberg-Konstante von 64S, und ist aus einem 45S- und einem 32S-Nukleoprotein--Komplex zusammengesetzt. Aus der 45S-Untereinheit lassen sich 5S RNA und zwei Formen 23S RNA isolieren, die sich in ihrer elektrophoretischen Mobilität unterscheiden. Die 32S-Untereinheit enthält die 16S RNA. Zwei Vorstufen der 16S

2. INTRODUCTION

The archaebacteria are a group of prokaryotes which are not more related to other bacteria (the true bacteria, or eubacteria) than to eukaryotic cells. In 1981, Woese and Gupta suggested that the archaebacteria represent a 'third urkingdom' in addition to the eubacteria and eukaryotes (119). This proposal rests on comparative sequence analysis of 16S (or 18S) rRNAs (33), and some other features at the molecular level. Other differences, besides those of the T1-RNA oligonucleotide catalogs of the 16S (or 18S) rRNAs (32,33), are the primary and secondary structure of 5S rRNA (52) and tRNAs (47), the nature and positions of post-transcriptional base modifications in 16S (or 18S) rRNA (120), and tRNAs, e.g. the lack of ribothymidine and 7-methylguanosine (47). In addition to those in RNAs, characteristic features of archaebacteria are 1. the structure of their DNA-dependent RNA polymerases which resembles that of eukaryotic RNA polymerases more than those of eubacteria (93,104,124), 2. the absence of a murein sacculus (57), 3. the presence of isopranyl ether in their lipids (23,62,63,64,108,109), 4. differences from eubacteria and eukaryotes in their ribosomal proteins and elongation factors for translation (59,76), 5. the occurrence of glycoproteins which are not found in eubacteria (77,123), 6. their unique ecology and special physiology, e.g. extreme thermophilia, acidophilia or halophilia, and the production

of methane or H_2S as a means of autotrophic existence, 7. their resistance to most antibiotics which block the growth of eubacteria, e.g. vancomycin, rifampicin, streptomycin and chloramphenicol (53,86,120).

Thus, archaebacteria differ strikingly from both eukaryotes and eubacteria in many molecular and physiological features. A number of fundamental properties important for the understanding of their function, e.g. their genome, gene organization and the modes of control of their gene expression, have not yet been analysed in comparison with the corresponding properties of eubacteria and eukaryotes. This thesis describes work on the organization of the rRNA structural genes (rDNA) of Thermoplasma acidophilum including an analysis of the rRNAs themselves. There are three reasons for the choice of rDNA for this type of analysis: 1. rDNAs are in all living cells, chloroplasts and mitochondria., 2. Their genes are among the best characterized. 3. Their sequences appear to be among the most highly conserved and therefore are particularly useful in taxonomic studies.

2.1 RIBOSOME AND rRNAs

Ribosomes, the protein synthesizing machines of all organisms, and in addition of chloroplasts and mitochondria, are composed of two ribonucleoprotein subunits differing in size. Two categories of ribosomes can be distinguished, namely the 70S and the 80S type. The 70S type of ribosome

exists in prokaryotes, chloroplasts and mitochondria, whereas the 80S type is found in the eukaryotic cytoplasm.

In the cytoplasmic ribosomes of rat liver, the best studied eukaryotic ribosomes, the small (40S) subunit contains one molecule of 18S rRNA of molecular weight 0.7×10^6. There are three rRNAs in the large 60S subunit, the 28S, 5.8S and 5S rRNA of molecular weights 1.7×10^6, 5.1×10^4 and 3.9×10^4 respectively (4,121).

The 16S rRNA of the small (30S) subunit of the E. coli ribosome comprises 1541 nucleotides (0.56x106 d.) (9); the 23S rRNA of the large (50S) subunit, 2904 nucleotides (1.07x106 d.) (10), the 5S of the 50S subunit rRNA 120 nucleotides (11). Other eubacteria including the blue-green algae have similar ribosomal components to E. coli. 5.8S rRNA has never been found in ribosomes of eubacteria and blue-green algae in contrast to those of eukaryotes.

The 23S, 16S and 5S rRNA of chloroplasts have molecular weights similar to those of eubacteria. However, the existence of some other rRNAs has been reported. Rochaix and Malnoë described 7S and 3S rRNA in Chlamydomonas chloroplasts (97); in flowering plants 4.5S rRNA has been found (27). The two large rRNAs of mitochondria, corresponding to the 16S and 23S rRNA in eubacteria, vary extremely in size among animals, ascomycetes, ciliated protozoans and higher plants (8). 5.8S rRNA has so far not been found in mitochondria. Except for those of higher plants, the mitochondrial ribosomes investigated so far lack

5S rRNA (46,65).

2.2 ORGANIZATION OF rDNA

The rDNA organizations of many eubacteria have been studied. Among these, E. coli and Bacillus rDNAs are especially well characterized. All the known eubacterial rDNAs have the typical structure of 5'-16S-23S-5S-3' (see Fig. 1). In the E. coli chromosome, there are at least seven, and probably only seven copies of this structure, the so-called rrn operons. The intercistronic region between the 16S and 23S rRNA genes contains either a gene for $tRNA^{Glu}$ or genes for both $tRNA^{Ile}$ and $tRNA^{Ala}$. Some other tRNA genes are located at the distal side of some rrn operons (80). Bacillus subtilis has 8-9 sets of rDNAs (79). Their structures resemble those of E. coli since both $tRNA^{Ile}$ and $tRNA^{Ala}$ also exist in the spacer of at least one (most likely two) of these rDNAs (69). The absence of tRNA genes has been reported for the other rDNAs of B. subtilis (14,92). The rDNA of Anacystis nidulans, a blue-green algae, has been recently cloned and analysed. It also has a structure similar to those of other eubacteria, and an unknown tRNA gene in the 16S-23S rRNA gene spacer (107).

The organization of eukaryotic rDNA has been intensively investigated in human (29,114), mouse (17), Xenopus (4,6,7,12,56,116), Drosophila (37,55,87, 113,115,117), Bombyx (75), Tetrahymena (28,58,118), Dictyostelium (16,34,72) and Saccharomyces (3,18,61,111), etc.. Redundancy

is a general phenomenon of eukaryotic rDNAs (68). The rDNAs are organized either in repeated sequence in one direction, e.g. Saccharomyces, or in palindromes, e.g. Tetrahymena, with or without intervening sequences in the 28S rRNA gene , e.g. Xenopus. Every single eukaryotic rDNA unit has the structure of 5'-18S-5.8S-28S-3'. While in higher eukaryotes the 5S rRNA genes are located at various chromosomal positions, in lower eukaryotes they are found down-stream of the 5'-18S-5.8S-28S-3' sequence, but both types of 5S rRNA gene generally have their own promotors, the so-called 5S DNA. The 5S rRNA is transcribed separately by RNA polymerase C in contrast to the large precursor RNA transcribed by RNA polymerase A from the 5'-18S-5.8S-28S-3' sequence.

The chloroplast rDNAs have a structure comparable to that found in eubacteria and blue-green algae, namely the 5'-16S-23S-5S-3' sequence. However, the spacer between the 16S and 23S rRNA genes can be as short as 400 bp, e.g. in Euglena gracilis ct-DNA which has the same structure as E. coli rrn operons with both the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes but not $tRNA^{Glu}$ (81,82), or it can be as long as 2.5 Kb, e.g. in Zea mays (2) or Chlamydomonas ct-DNA (73,96,97). The chloroplasts may have only one set, e.g. Vicia faba (60), two sets in inverted direction, e.g. Chlamydomonas and Pennisetum (94), or three sets of rDNA repeated in one direction per ct-genome, e.g. Euglena gracilis.

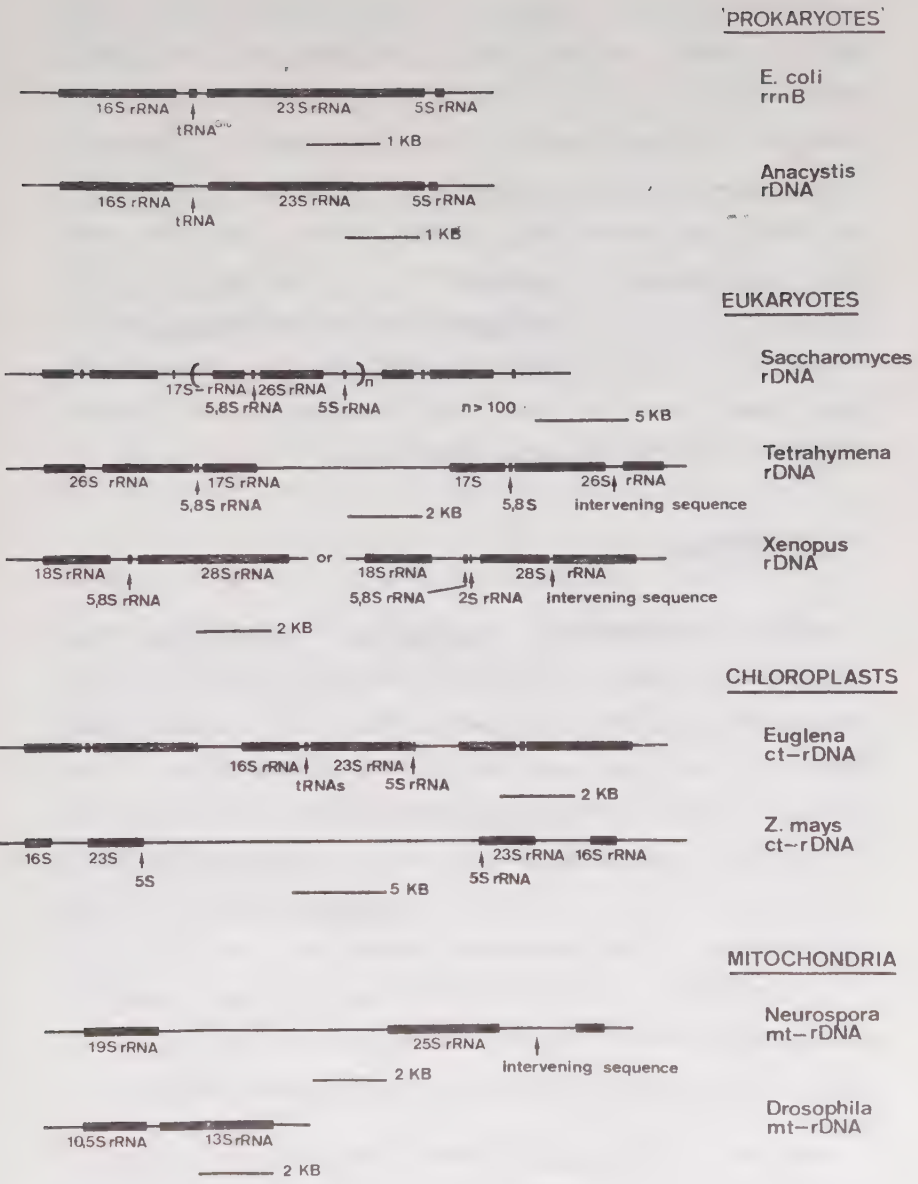

Fig. 1 rDNA organization of some eubacteria, eukaryotes, chloroplasts, and mitochondria.

The mitochondrial genomes of many organisms have been shown to contain one copy each of the large and the small rRNA genes. In all mitochondrial DNA of animal cells so far examined, the genes encoding the two rRNAs are closely linked and separated by a spacer of 100-200 bp (122). In contrast to the situation in animal cells, the two rRNA genes in Neurospora are separated by a sequence of 5 Kb (24,48,52,74), and in Tetrahymena and Saccharomyces by 10 (38,39) and 30 Kb (31,100) respectively. In some known cases, tRNA genes or tRNA gene cluster(s) are located between the two mitochondrial rRNA genes.

2.3 THERMOPLASMA

The thermoacidophile Thermoplasma acidophilum was isolated by Darland et al. from burning coal refuse piles. The cells are pleomorphic spheres, vary in diameter from about 0.3-2 µm, and lack a rigid cell wall (20). The molecular weight of Thermoplasma genome is $1x10^9$, the smallest yet reported for any nonparasitic organism, with a G+C content of about 46% (15). Thermoplasma belongs to the archaebacteria in all described aspects, and is classified as an independent genus, or even order on the basis of partial sequencing of the 16S rRNAs (32) and of the cross hybridization between 16S rRNAs and chromosomal DNAs (110).

Some work on Thermoplasma RNAs other than the 16S rRNA T1 oligonucleotide catalog(32) has been reported. The initiator tRNA was sequenced (112). The base modifications and loop

structures show some typical eubacterial but also eukaryotic features. It has no more than 70% homology with any other initiator tRNA so far described. The primary structure of Thermoplasma 5S rRNA shows only 60.6% homology with that of Halobacterium, another archaebacterium, and even less with others. Its secondary structure does not conform to usual models employed for either prokaryotic or eukaryotic 5S rRNA. However, the loop structures of Thermoplasma 5S rRNA appear more eukaryotic than eubacterial (70).

Thermoplasma was chosen as the experimental organism because 1. Its cytoplasmic condition is not very extreme, e.g. the salt concentration in the cell is 'normal' as opposed to that in Halobacteria, and the optimal growth temperature is not as high as that of other thermophiles. Therefore it can be compared reasonably with eubacteria and eukaryotes. 2. The Thermoplasma RNA polymerase is one of the best and most intensively studied archaebacterial RNA polymerases. The combination of gene isolation and RNA polymerase research could lead to the understanding of archaebacterial transcription.

3. RESULTS

3.1 ANALYSIS OF THERMOPLASMA RIBOSOMES AND RNAs

3.1.1 RIBOSOMES

To analyse the S-values of ribosomes and ribosomal subunits, Thermoplasma cells were labeled for 10 min with ^{32}P, lysed and analysed by sucrose gradient centrifugation as described in METHODS.

Thermoplasma ribosomes and their subunits showed three peaks in sucrose gradients (see Fig. 2 and Fig. 3). Fig. 2 shows that these have sedimention coefficients of 64S, 45S and 32S respectively, when E. coli ribosomes are used as standards.

The Mg^{++} requirement for the stability of Thermoplasma ribosomes was tested. In the range of 10-100 mM Mg^{++} no 64S peak was visible at 260 nm (data not shown). when Thermoplasma cells were labeled with ^{32}P for 20 min, however, the 64S ribosome peak could clearly be recognized as well as the 45S and 32S subunit peaks.

3.1.2 THERMOPLASMA RNAs

For convenience, the stable rRNAs in Thermoplasma are named 23S, 16S and 5S rRNA as in the E. coli system, even though their exact S-values have not been determined.

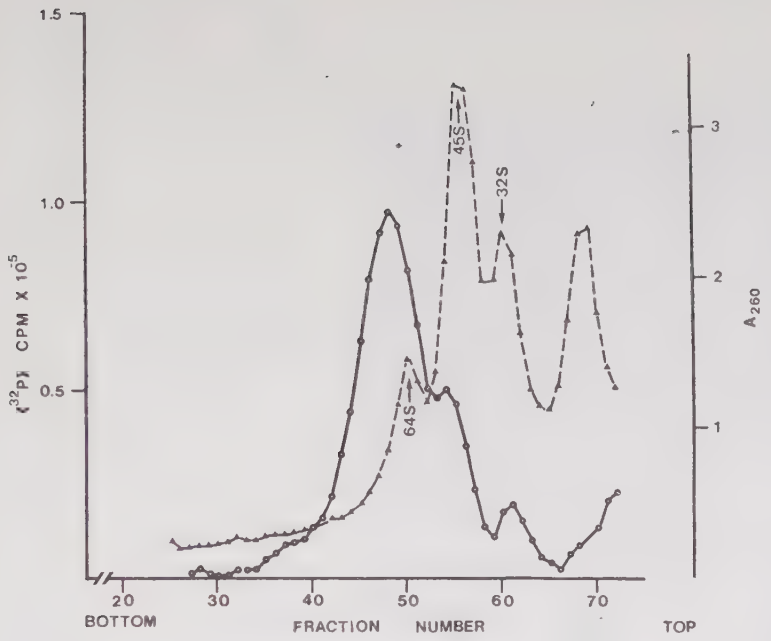

Fig. 2 S-value analysis of Thermoplasma ribosomes.

Cell lysate of ^{32}P-labeled Thermoplasma (triangles, cpm) was fractionated in sucrose gradient centrifugation (10-30% sucrose, SW 41, 35,000 rpm for 3 hr at 15°C). Purified E. coli ribosomes (opened circles, A_{260}) were used as standards.

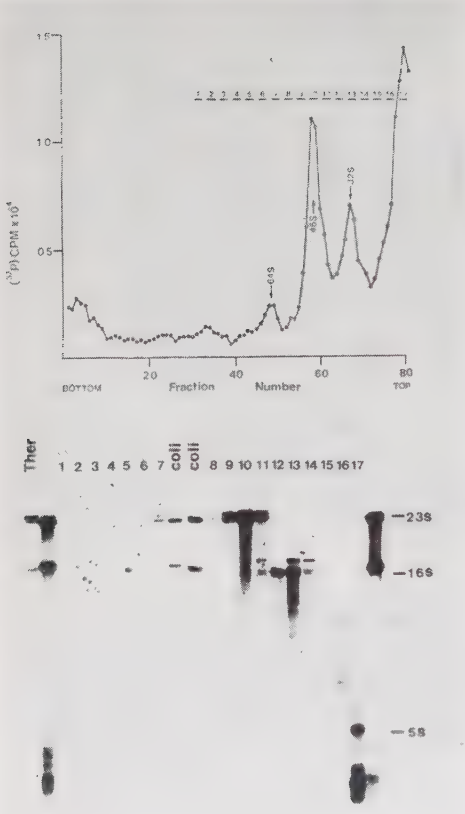

Fig. 3 RNA composition of Thermoplasma ribosomes.

RNA species (lower) in the given ^{32}P-labeled Thermoplasma ribosome fractions (upper) were analysed in a 2% acidic urea agarose gel.

(1) RNA COMPOSITION OF THERMOPLASMA RIBOSOMES

The RNA composition of fractions of ^{32}P-labeled Thermoplasma cell lysates separated by sucrose gradient centrifugation was analysed by gel electrophoresis. Before analysis, fractions (see Fig. 3 upper) were extracted with phenol and precipitated with ethanol in the presence of E. coli tRNA as carrier. Fig. 3 shows the presence of different RNA species in these fractions. The 64S ribosomes (Fig. 3, track 7) and the polysomes (tracks 1-5) contain four species of rRNAs, namely two 23S rRNAs, one 16 rRNA and a 5S rRNA (The radioactivities of 5S rRNA and mRNA in 64S ribosomes and polysomes are too weak to be seen in Fig. 3). As expected the 16S rRNA is absent in the 45S subunit (track 10), whereas in the 32S subunit (track 13) it is present and accompanied by two strong bands with slightly lower mobilities and some mRNAs between the positions of 16S rRNA and 5S rRNA. The large (45S) subunit contains 5S rRNA and the two 23S rRNAs. The existence of mRNAs in the 32S ribosome subunit means that the 32S subunit has the same ability to bind mRNAs as the small ribosomal subunit in other organisms.

(2) MOLECULAR WEIGHTS OF rRNAs

To determine the molecular weights of the rRNAs, ^{32}P-labeled rRNA was used. Thermoplasma cells were labeled for 20 min and lysed. Total RNAs and RNAs extracted from ribosomes were analysed in an acid-urea agarose gel using rRNAs from S. cerevisiae and E. coli as standards.

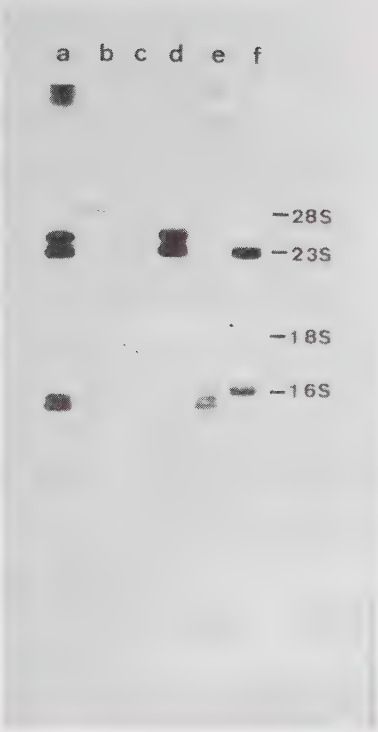

Fig. 4 Molecular weight determination of Thermoplasma rRNAs.

rRNAs larger than 5S from 64S ribosomes (track a), 45S (track d) and 32S subunits (track e) were analysed by 2% acidic urea agarose gel electrophoresis. rRNAs from E. coli 70S ribosomes (track f), S. cerevisiae 80S ribosomes (track b) and 40S subunits (track c) were used as standards.

Fig. 4 shows the mobilities of these rRNAs. The two 23S rRNAs of Thermoplasma have an apparent molecular weight of 1.2×10^6 and 1.1×10^6 respectively. The three dominant RNAs in the 32S subunit, i.e. the 16S rRNA and the two larger RNAs, appear in the range of E. coli 16S rRNA. They have molecular weights of 0.48×10^6, 0.51×10^6 and 0.59×10^6 respectively.

(3) rRNA PRECURSORS (p-rRNAs)

As described above two 23S and one 16S rRNAs can be found in mature Thermoplasma ribosomes; the two additional RNAs larger than 16S rRNA exist only in free 32S subunits. In order to investigate possible precursor relationships, the techniques of chase experiment and of homology hybridization were used.

By adding an excess of cold H_3PO_4 after labeling and continuing the growth, the unstable precursors can be chased. Fig. 5 shows that both 23S rRNAs and the 16S rRNA are nonchaseable, whereas the other two RNAs found in 32S subunits are unstable.

The five (r)RNAs were eluted from the gel and hybridized individually to restriction enzyme-digested Thermoplasma DNA which had been transferred by Southern blotting onto nitrocellulose sheets after electrophoretic separation of the fragments. The hybridization data (Fig. 6) show that these five (r)RNAs belong to two RNA groups which hybridize to different sets of restriction fragments.

Both 23S rRNAs hybridized to one fragment of Cla

I-digested DNA (6.6 Kb) and two fragments of Bgl I-(12.4 and 3.7 Kb) and Hind III (2.7 and 0.73 Kb) (Fig. 6). In contrast, the 16S rRNA and its two satellites hybridized to only one band of Bgl I-, Cla I- and Hind III- digested DNA (2.9, 5.5 and 2.1 Kb respectively). Therefore these two groups of rRNA are each transcribed from their own DNA sequence in the chromosome. The RNA hybridizing DNA fragments marked with A are artifacts. During Thermoplasma rRNA preparation, smaller rRNAs (including 5S rRNA) are usually contaminated by broken larger rRNAs (possibly by enzymatic breakdown, see THE COPY NUMBER OF rRNA GENES IN THERMOPLASMA). Since the hybridization experiments were usually done by adding an excess of hot rRNA probes, any such contamination would result in artifactual hybridization to DNA fragments. This can be avoided by adding a large amount of the contaminating rRNA in cold form.

Thus the two unstable RNAs in the 32S ribosomal subunit appear to be precursors of the 16S rRNA. The larger of the two 23S rRNAs cannot be the precursors of the smaller one, since both are transcribed from the same DNA fragment and neither is chaseable. The 23S rRNA of the molecular weight of 1.2×10^6 should be the only true 23S rRNA in Thermoplasma ribosomes. The 'smaller' one (1.1×10^6 d.) may be an artifact caused by the secondary structure of the 'larger' one (see DISCUSSION).

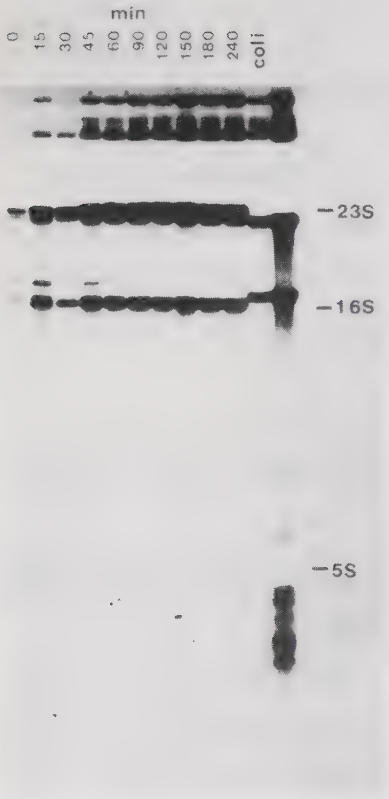

Fig. 5 Chase experiment of Thermoplasma rRNAs.

Thermoplasma cells were labeled with $H_3{}^{32}PO_4$ (25 µCi/ml, carrier free) for 15 min. After adding excess H_3PO_4 (10mM) to the medium, samples were taken at 0, 15, 30, 45, 60, 90, 120, 150, 180 and 240 min.

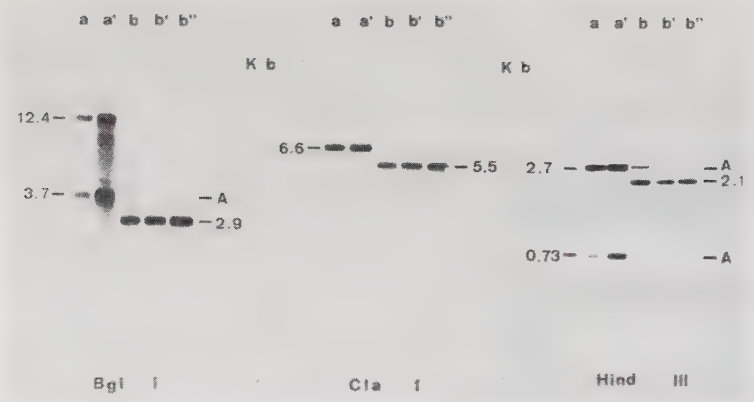

Fig. 6 Hybridization of 23S rRNA, 16S rRNA and the assumed p-rRNAs to Bgl I, Cla I and Hind III digested Thermoplasma DNA.

Lane a and a' are the two 23S rRNAs (molecular weight 1.2×10^6 and 1.1×10^6 respectively). Lanes b, b' and b" are the stable 16S rRNA and its two precursors (b', 0.59×10^6; b", 0.51×10^6).

3.2 ANALYSIS OF THERMOPLASMA rDNA

3.2.1 THE COPY NUMBER OF rRNA GENES IN THERMOPLASMA

To determine the rRNA gene copy number in Thermoplasma, nitrocellulose filters containg 7-10 µg of DNA were incubated separately with logarithmically increasing amounts of rRNAs (sp. act. about 4 µCi/µg), until the saturation plateau had been reached (see METHODS). After hybridization the nonspecifically bound rRNA was removed by RNase digestion. The remaining radioactivity on the filters was estimated in a liquid scintillation counter and the corresponding weights were calculated.

Fig. 7 shows the saturation hybridization curves for 23S and 16S rRNAs. The hybridization of 23S rRNA to Thermoplasma DNA reaches a saturation plateau at about 11 ng RNA to 10 µg DNA. The molecular weights of 23S rRNA and Thermoplasma DNA are about 1.2×10^6 and 1×10^9 respectively. The result shows clearly that there is only one gene copy for 23S rRNA.

Hybridization of 16S rRNA to DNA results in a two-phase saturation curve. The first plateau is reached at 6.5 ng RNA to 10 µg DNA, while final saturation is reached at more than 17 ng RNA to 10 µg DNA. This two-step saturation curve has appeared in all repeated experiments. It can be explained by the previously described contamination of the 16S rRNA by the 23S rRNA. The lower plateau is due to 16S rRNA with a small amount of contaminating 23S rRNA. After saturation of 16S rRNA, further hybridization is due to increasing amounts

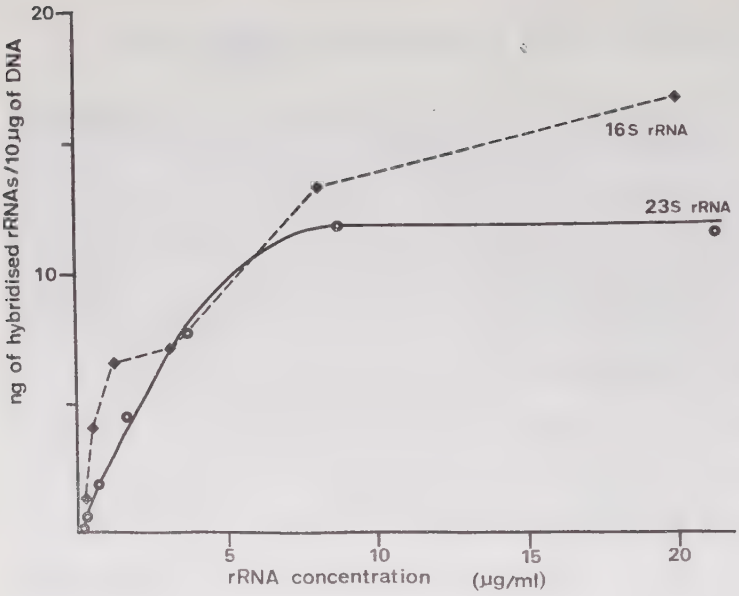

Fig. 7 Saturation hybridization of Thermoplasma DNA with 16S and 23S rRNAs.

Filters containing 7-10 µg of DNA were hybridized with logarithmically increasing amounts of ^{32}P-labeled 16S or 23S rRNA for 16hr at 60°C. After RNase digestion the radioactivities of 16S (diamonds) and 23S (opened circles) on the filters were determined by Cerenkov counts.

of contaminating 23S rRNA alone. If the first plateau is related to 16S rRNA, then this gene should also be present as a single copy. The final saturation level is consistent with hybridization of the DNA by one molecule each of the 16S and 23S rRNA per genome. In addition, the presence of multiple gene copies should not lead to multiple-phase saturation hybridization.

The contamination of smaller rRNAs by larger RNAs has been demonstrated and will be discussed later. To overcome this problem, subsequent experiments were carried out by adding an excess of the contaminating rRNA(s) in cold form to chase the nonspecific hybridization. However, in this experiment the chasing procedure was not used because of the solubility of the rRNA. Saturation is only achieved in the presence of excess labeled probe so that correspondingly higher amounts of cold 23S rRNA would be required. The 5S rRNA saturation hybridization has been done in the same way (data not shown). Owing to the small size of the 5S rRNA (1/12 and 1/25 of those of 16S and 23S rRNA respectively), the radioactivities of the contaminating 16S and 23S rRNAs influenced strongly the determination sensitivity. The first saturation plateau was not detectable. It is presumed that there is also only one gene copy for 5S rRNA.

3.2.2 rDNA ANALYSIS IN THE THERMOPLASMA CHROMOSOME

To determine the structural relationship between the 23S, 16S and 5S rRNA genes, Thermoplasma DNA was digested separately with the restriction enzymes Bam HI, Bgl I, Cla

I, Eco RI, Hind III and Pst I. The restriction fragments were separated in 1% agarose gels by electrophoresis, and transferred to DBM-paper (see METHODS). Purified 5'-^{32}P-labeled rRNAs were hybridized to the DBM-paper sheets. The hybridization results are shown in Fig. 8, and summarized in Table 1.

Table 1. Sizes (Kb) of Thermoplasma DNA restriction fragments which hybridize to rRNAs

enzyme used for digesting Thermoplasma DNA	rRNA used for hybridization		
	23S	16S	5S
Bam HI	2.7, 0.83	6.7	4.8
Bgl I	12.4, 3.7	2.9	11.2
Cla I	6.6	5.5	7.2
Eco RI	9.7	5.3	6.8
Hind III	2.7, 0.73	2.1	1.3
Pst I	3.7, 3.3	9.3	1.5

The results show 1. The 23S rRNA gene is digested into two fragments by Bam HI, Bgl I, Hind III and Pst I, whereas it remains intact in the Cla I and Eco RI digests. 2. 16S and 5S rRNA each hybridizes to one fragment only in each of the digests. 3. No apparent heterogeneous gene structure in any of the three rRNA genes can be found. 4. There is no DNA fragment hybridizing with two of the three rRNAs, i.e. no linkage relationship between 23S, 16S and 5S rRNA genes can be found in any of the six digests.

There are only two explanations for the fourth

Fig. 8 Hybridization of ^{32}P-labeled Thermoplasma 23S, 16S and 5S rRNAs to restriction endonuclease digested DNA.

Thermoplasma DNA was digested separately with Bam HI, Bgl I, Cla I, Eco RI, Hind III and Pst I. The digests were fractionated by electrophoresis in 1% agarose gels and transferred onto DBM-paper. The sheets were hybridized to 5'-^{32}P-labeled rRNAs. Hind III digested T5 phage DNA fragments were used as size standard.

observation: 1. Each of the three rRNA genes is far apart from the others, and the sizes of the spacers are larger at least than the average fragment size generated by these six enzymes, i.e. 4.1 Kb (4^6 bp). In this situation, restriction enzymes whose recognition sequence is 6 bp should have on average at least one cleavage site in the spacers. 2. The spacers are relatively short, but could have very special palindromic sequences which can be restricted by many restriction endonucleases.

Examination of the sizes of the hybridized DNA fragments listed in Table 1 suggests that the first explanation is correct. The sum size of all Thermoplasma rRNA genes (assuming all three genes are in one operon) must be larger than 15 Kb, no matter what the gene order is. For example, the 5S rRNA hybridizes to an 11.2 Kb Bgl I fragment. Even if the 23S and 16S rRNA genes lie just beyond the ends of the Bgl I fragment to which 5S rRNA hybridizes, the total size of the operon must be greater than 15 Kb assuming that the 5S rRNA gene lies between the 23S and 16S ones. If however the 16S or 23S rRNA gene lies in the middle, a similar argument can be made from the Pst I and Eco RI digests respectively.

3.2.3 CLONING OF THERMOPLASMA rDNA

(1) CLONING

In order to analyse Thermoplasma rDNA structure more clearly, the rDNA was cloned in pBR322. The restriction

enzymes Bam HI and Hind III were chosen to clone the Thermoplasma rDNA (see METHODS). The ratio of restricted Thermoplasma DNA to pBR322 vector DNA was 1:1 (w/w) for ligation. Because Thermoplasma has a genome size of about 1.5×10^6 bp (ca. 1×10^9 d), and both restriction enzymes cut DNA into fragments with an average size of 4.1 Kb (4^6 bp), the Thermoplasma genome should be restricted into 350 fragments by the enzymes. There is one restriction site in pBR322 for both Bam HI and Hind III, and the genome size of pBR322 is about 1/350 of that of Thermoplasma, i.e. 4362 bp. Thus a weight ratio of 1:1 is a molar ratio of about 1:1.

After transformation, the C600 cells were diluted serially, and plated on LB-agar with or without either ampicillin or tetracycline to calculate the efficiencies of transformation. The results are shown in Table 2.

Table 2. Efficiencies of shotgun cloning of restricted Thermoplasma DNA into pBR322 (conditions see METHODS)

	enzyme used for cloning	
Efficiencies	Bam HI	Hind III
transformants/viable cells	1.4×10^{-4}	1×10^{-4}
transformants/µg of vector	6.5×10^4	3.9×10^4
clones/transformants (%)	26	28

For high density plasmid screening the transformed cells were plated directly onto sterilized nitrocellulose sheets on LB agar plates containing ampicillin. The survival efficiency compared with plating the cells on LB agar plates

without nitrocellulose is about 10%.

(2) ISOLATION OF rDNA CLONES

Hybridization with rRNAs was the only method used to detect rDNA clones. The rDNA clones were isolated by high density plasmid screen and selection (see METHODS). Before plasmid DNA preparation, the isolated clones were tested using a mini-lysate technique (see METHODS). Plasmids were released by treating the cells harvested from 1 ml of overnight culture with SDS. Proteins and RNAs were removed by phenol extraction and RNase digestion. After RNase digestion, the plasmids were digested with Bam HI or Hind III (depending on the clone batch).

To confirm the types of clones, the restriction and hybridization patterns were compared after gel electrophoresis, Southern blotting and hybridization. From 42 rDNA clones (Bam HI, 25; Hind III, 17) five different types were isolated (see Table 3). Three from the Bam HI batch contain plasmid carrying fragment(s) of 23S rDNA, the pTB82, pTB52 and pRB224. One of the two Hind III clones is a 23S rDNA clone containing pTH1101, whereas the other is a 16S rDNA clone containing pTH 126.

Table 3. Plasmids hybridizing rRNAs

	Bam HI batch			Hind III batch	
rRNA	pTB82	pTB52	pRB224	pTH1101	pTH126
23S	+	+	+	+	-
16S	-	-	-	-	+

3.2.4 ANALYSIS OF THE CLONED rDNA FRAGMENTS

After the cloning of the Thermoplasma rDNA, the structures of the recombinant plasmids, i.e. the orientations of the inserted fragments, were analysed by using a combination of Bam HI and Hind III digestions (single digestions of each and double digestion). The inserted rDNA fragments were mapped with the restriction enzymes Bgl I, Cla I, Eco RI , Pst I and Taq I. To determine the positions of rRNA genes in the cloned DNA fragments, purified 5'-^{32}P-labeled rRNAs were used to hybridize the electrophoretically separated Taq I-digested fragments of the plasmids.

(1) STRUCTURE OF THE PLASMIDS

The structures of the plasmids carrying Thermoplasma rDNA were analysed by digesting each of these five plasmids with 1. Bam HI, 2. Hind III, and 3. Bam HI + Hind III. Fig. 9 shows the restriction patterns of these plasmids. The pBR322 restriction cleavage map is shown in Fig. 10. The cleavage coordinates for some restriction enzymes are listed in Table 4.

In pBR322, the distance between the Bam HI and Hind III restriction sites is 355 bp. The Bam HI digested plasmids which contain rDNA Bam HI fragments, and Hind III digested plasmids which contain rDNA Hind III fragments, release pBR322 (4.4 Kb, marked with stars(★))and the inserted fragments (see all tracks A). By reciprocal digests it can

be shown whether there is a Hind III site in a given Bam HI cloned fragment, and vice versa. Using the positions of these restriction sites the orientations of the cloned fragments can be determined by comparison with those (of the Bam HI and Hind III sites) in pBR322. The Hind III digested pTB82 (B1 in Fig. 9) shows no Hind III site in the inserted fragment. The Hind III digested pTB52 (B2 in Fig. 9) and the Bam HI digested pTH126 (B5 in Fig. 9) show a Hind III and a Bam HI site in the inserted fragment of pTB 52 and of pTH126 respectively. B4 and B3 in Fig. 9 show two Hind III and three Bam HI sites in the cloned fragments of pRB224 and pTH1101 respectively. In double digestions (all tracks C in Fig. 9), all restriction fragments including the two bands of pBR322 (4007 and 355 bp, marked with stars (★)) are released.

Table 4. The cleavage coordinates in pBR322 for enzymes which are used in this thesis

Enzyme	cleavage coordinate(s)
Bam HI	375
Bgl I	934, 1168, 3487
Cla I	24
Eco RI	4360 (or -2)
Hind III	29
Taq I	23, 338, 650, 1125, 1266, 2573, 4017
Pst I	3612

The numbers correspond to where the restriction endonuclease nicks the strand which is 5' to 3' in the clockwise direction (ref. 105).
The size of PBR322 is 4362 bp.

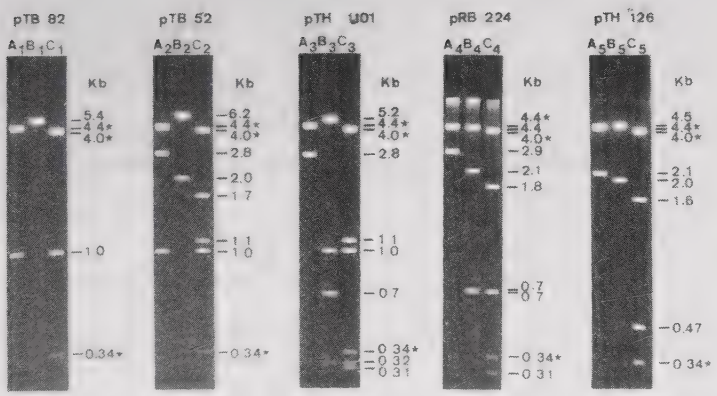

Fig. 9 Gel electrophoresis of Bam HI, Hind III, and Bam HI-Hind III double digests of the plasmids carrying Thermoplasma rDNA.

pTB82, pTB52 and pRB224 were digested with Bam HI (A1, A2 and A4), Hind III (B1, B2 and B4) and Bam HI + Hind III (C1, C2 and C4). pTH1101 and pTH126 were digested with Hind III (A3 and A5), Bam HI (B3 and B5) and Bam HI + Hind III (C3 and C5). Fragments were electrophoretically separated in 2% agarose gels.

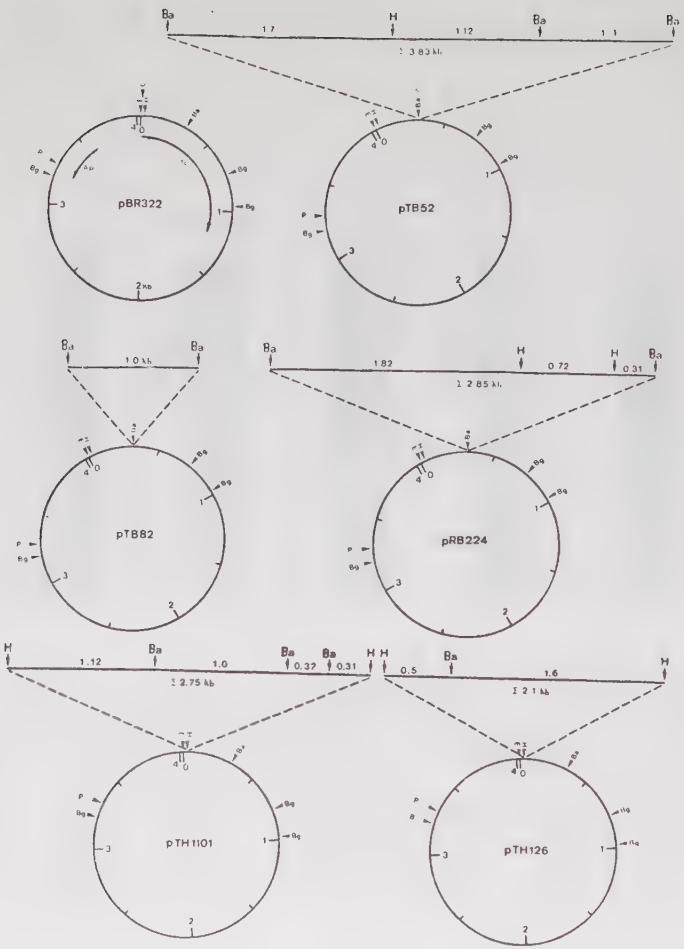

Fig. 10 Schematic representations of pBR322 and the Thermoplasma rDNA-carrying plasmids pTB82, pTB52, pRB224, pTH1101 and pTH126.

The schemes show the insertion positions and the sizes (Kb) of the inserts.

By calculating the molecular weights of the fragments in all three digests , the structures of the plasmids can be constructed. The plasmid structures and the sizes of the inserted fragments are shown in Fig. 10.

pTB52 has two inserted fragments due to partial digestion during cloning (A2 in Fig. 9). pTH1101 has three Bam HI restriction sites in its cloned fragment (B3 in Fig. 9). The continuity of the two fragments of pTB52 and the exact position of the middle Bam HI site in pTH1101 were shown in the next experiments.

(2) RESTRICTION MAPPING OF THE CLONED FRAGMENTS

(A) MAPPING BY BGL I, CLA I, ECO RI AND PST I DIGESTION

The cloned Thermoplasma rDNA fragments were mapped with the restriction enzymes Bgl I, Cla I, Eco RI and Pst I. The plasmids cloned with Bam HI were digested by 1. one of these four enzymes, 2. that enzyme + Bam HI. For the plasmids cloned with Hind III, Hind III was used instead of Bam HI in the double digestion. Fig. 11 shows the digestion patterns (data for pTB82 not shown). The restriction maps of all cloned fragments are shown in Fig. 12. The maps were constructed as described above.

In each of pTB82, pTB52 and pTH1101, there is a Pst I site. By calculating the distances between this and the Bam HI sites to either side, and the distance between the Pst I site and the Hind III sites to its left side in pTB52 and pTH1101, it becomes clear that the inserts in pTB82, pTB52

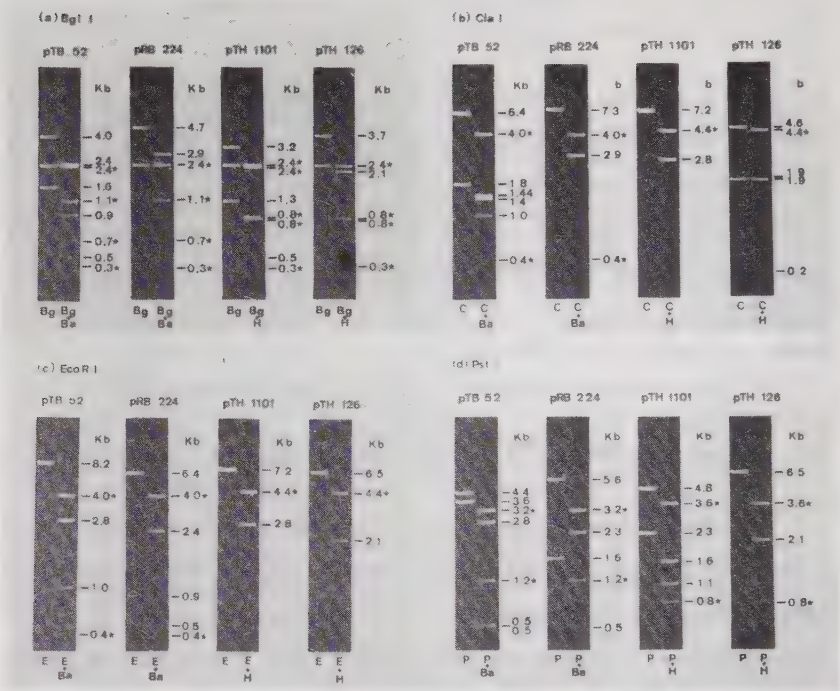

Fig. 11 Restriction cleavage patterns of the plasmids carrying Thermoplasma rDNA by (a) Bgl I, (b) Cla I, (c) Eco RI and (d) Pst I in the presence and absence of Bam HI or Hind III.

Ba, Bg, C, E, H, and P are Bam HI, Bgl I, Cla I, Eco RI, Hind III and Pst I respectively. Fragments containing only pBR322 sequences are marked with stars (★). Fragments were fractionated by electrophoresis in 2% agarose gels.

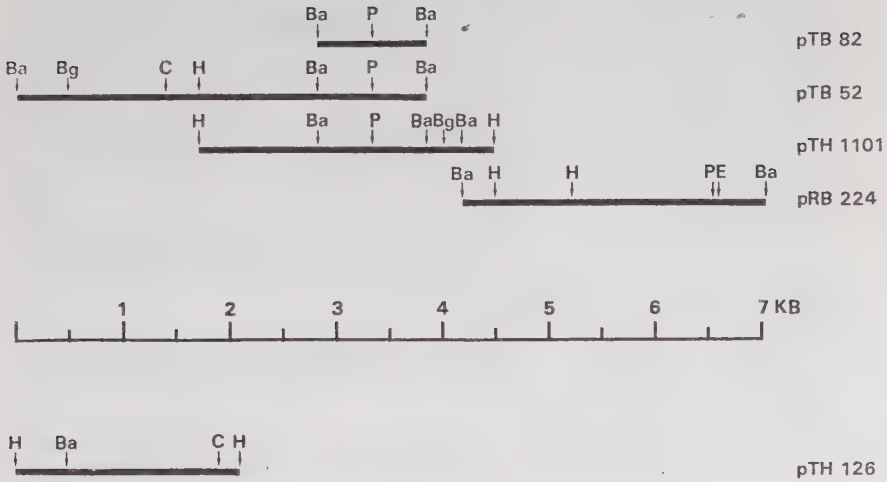

Fig. 12 Physical maps of the cloned Thermoplasma rDNA fragments. Bam HI (Ba), Bgl I (Bg), Cla I (C), Eco RI (E), Hind III (H) and Pst I (P).

and pTH1101 are overlapping, and that the two fragments in pTB52 are continuous in the genome. There is another Pst I site and an Eco RI site in pRB224, a Bgl I site in each of pTB52 and pTH1101 and a Cla I site in pTB52. The distance between the two Pst I sites is about 3.2 Kb, whereas that between the two Bgl I sites is about 3.5 Kb. In pTH126 there is only a Cla I site on the other side of the Bam HI site.

The overlapping of pRB224 and pTH1101 is consistent with the distance between the Hind III and Bam HI sites at the ends of the overlapped fragments, and with the data from Taq I-mapping and hybridizations (see below). The insert in pTH126 does not overlap with any other cloned fragment.

(B) TAQ I-MAPPING

In Taq I-mapping each of the plasmids was digested by 1. Taq I, 2. Taq I + Bam HI, 3. Taq I + Hind III, and 4. Taq I + Bam HI + Hind III. The restricted fragments were electrophoretically separated in polyacrylamide-agarose composite gels (see METHODS). Fig. 13 shows the restriction patterns of vector pBR322, pTB82, pTB52, pRB224, pTH1101 and pTH126 respectively. The molecular weight standards were Hae III- and Alu I-digested pBR322. The restriction fragment sizes of the cloned DNA are given in bp. The bands which were altered or created by adding a further enzyme are connected by arrowed lines. Fragments containing only pBR322 sequences are marked by stars (*); fragments containing both sequences of pBR322 and cloned Thermoplasma DNA are marked by double stars (**). Fig. 14 shows the Taq I maps of pTB82,

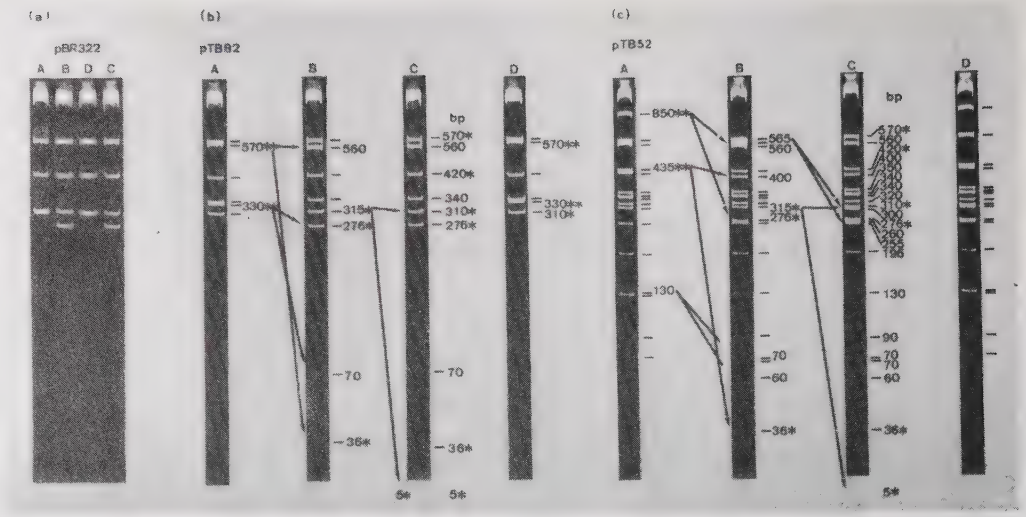

Fig. 13 Analysis of the Taq I digests of the plasmids carrying Thermoplasma rDNA.

The plasmids were digested with 1. Taq I (A), 2. Taq I + Bam HI (B), 3. Taq I + Hind III (D) and 4. Taq I + Bam HI + Hind III (C). Fragments were electrophoretically separated in 5% polyacrylamide-0.5% agarose composite gels. (a), (b), (c), (d), (e) and (f) show the digests of pBR322, pTB82, pTB52, pTH1101 and pTH126 respectively. The bands which were altered or created by adding a further enzyme are connected by arrowed lines. Size standards were Hae III and Alu I digested pBR322 fragments. * denotes the fragments containing only pBR322 DNA sequence. ** denotes the fragments containing both sequences of pBR322 and of cloned Thermoplasma DNA.

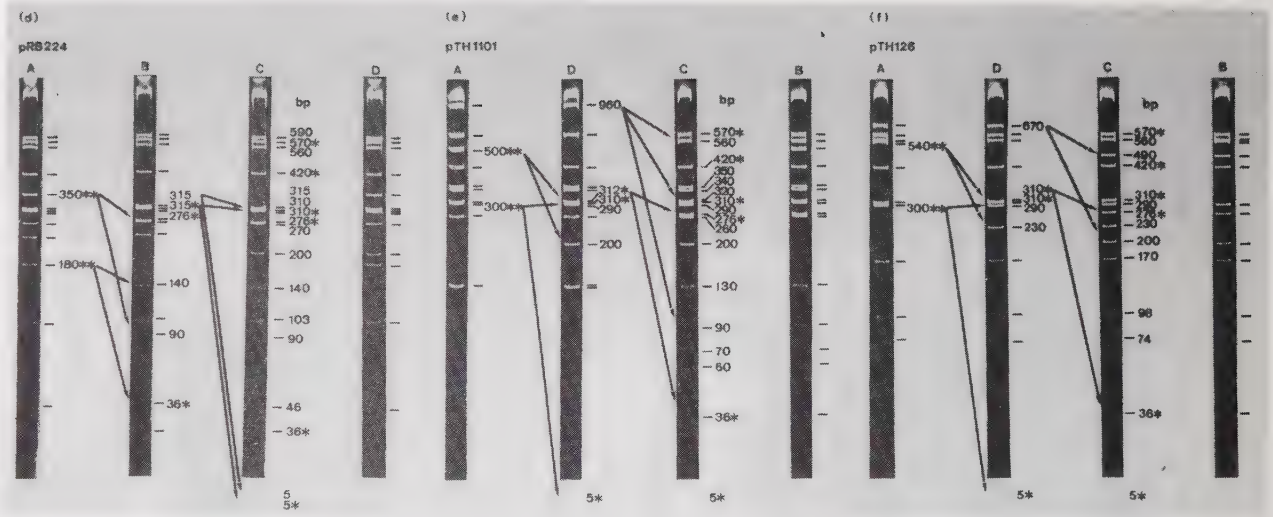

Fig. 13 Analysis of the Taq I digests of the plasmids carrying Thermoplasma rDNA. (d), (e) and (f).

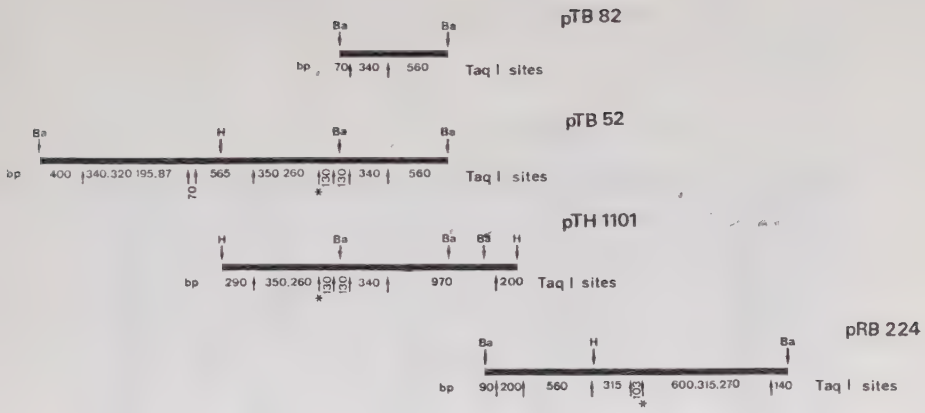

Fig. 14 Physical maps of the Taq I restriction sites on the cloned Thermoplasma 23S rDNA fragments.

* denotes the Taq I sites which have been identified after rRNA-DNA hybridization (see Fig. 16). Some Taq I sites are still unmapped. The sizes of the corresponding fragments are shown between the neighboring known sites.

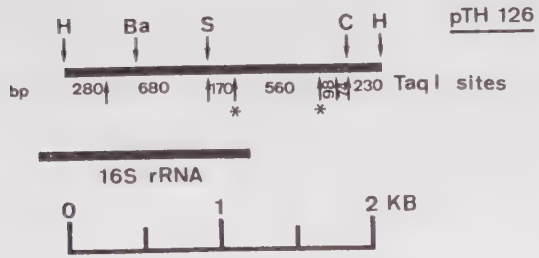

Fig. 15 The physical map of Taq I restriction sites on the cloned Thermoplasma 16S rDNA and the corresponding position of the 16S rRNA gene.

* denotes the Taq I sites which have been identified after rRNA-DNA hybridization (see Fig. 16). The sizes of the corresponding fragments which are flanked by two unidentified Taq I cleavages are shown between the neighboring known sites.

pTB52, pTH1101 and pRB224. The Taq I map of pTH126 is shown in Fig. 15.

Taq I sites, neighboring both ends of the cloned fragments, neighboring a Bam HI or a Hind III site, or which are also sites for Cla I or Sal I sites could be recognized (Cla I and Sal I have a 6-base recognition site. Taq I recognises the internal 4 bases of both). The mapping order of the cloned fragments hybridizing with 23S rRNA must be pTB82-pTB1101$<^{pRB224}_{pTB52}$, since their sizes and their overlapping relationship. Some Taq I sites are still unmapped. The sizes (in bp) of the corresponding fragments are shown between the neighboring known sites. All sites in the rRNA genes and beside them however have been identified.

(3) POSITIONS OF rRNA GENES IN THE CLONED FRAGMENTS

All plasmids were triply digested by Taq I, Bam HI and Hind III. The restriction fragments were transferred to DBM-paper after separation by electrophoresis in composite gels (see METHODS), and hybridized using purified 5'-^{32}P-labeled rRNAs and tRNAs. Fig. 16 shows the comparison between the restriction fragment and hybridization patterns. The reconstituted regions covered by the cloned fragments and the corresponding positions of 23S rRNA gene and of the single cloned fragments are shown in Fig. 17. The data for 16S rRNA gene are shown in Fig. 15.

The overlapping inserts of the pTB82, pTB52, pTH1101 and pRB224 cover a DNA fragment with a size of more than 7 Kb.

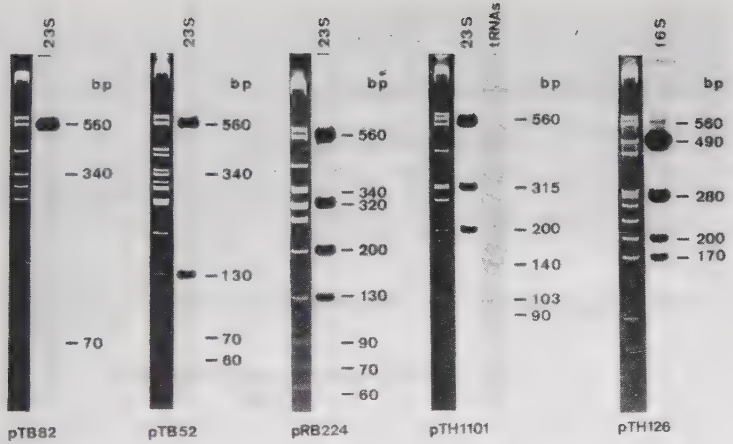

Fig. 16 Hybridization of the restriction digests of the plasmids carrying Thermoplasma rDNA with 5'-^{32}P-labeled rRNAs and tRNAs.

The plasmids were triply digested with Taq I, Bam HI and Hind III. Only the rRNAs and tRNAs which gave positive hybridization are shown.

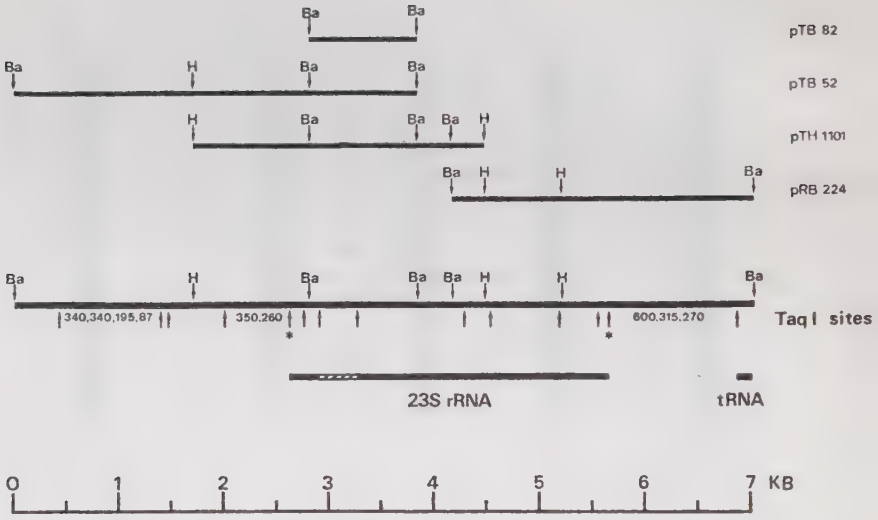

Fig. 17 Corresponding positions of 23S rRNA and tRNA in the cloned the Thermoplasma 23S rDNA fragments.

* denotes the Taq I sites which have been identified after RNA-DNA hybridization. The sizes of the corresponding fragments which are flanked by two unidentified Taq I cleavages are shown between the neighboring known sites.

This DNA fragment contains an entire 23S rRNA gene of about 3 Kb, and a tRNA gene or a part of it at one terminus of the covered region. The distance between 23S rRNA gene and the tRNA gene is about 1.2 Kb. In this spacer and in the DNA sequence on the other side of the 23S rRNA gene, no detectable 16S or 5S rRNA gene has been found. The pTH126 has an insert about 2.1 Kb in size which contains most of the 16S rRNA gene, i.e. 1.2 Kb. No 23S, 5S rRNA gene or tRNA gene sequence has been found in the rest of this cloned DNA fragment.

3.2.5 LINKAGE RELATIONSHIP ANALYSIS OF rRNA GENES IN THE THERMOPLASMA GENOME USING THE PLASMIDS CARRYING rDNA

Since the whole 5S rRNA gene and a small part of 16S rRNA gene are missing during rDNA cloning, and although the whole cloned regions are large enough to discover close linkages, such a linkage between the three rRNA genes has not been found. Using the nick-translated plasmids to detect the linkages between the rRNA genes or at least the distances between them became necessary. The hybridization of nick-translated plasmids to electrophoretically separated Bgl I, Cla I, Eco RI and Pst I fragments of Thermoplasma DNA can expand the analysable regions into the regions flanking the cloned DNA sequences up to the next sites for these restriction enzymes (remember that the rDNA has been cloned with Bam HI and Hind III).

Bgl I, Cla I, Eco RI and Pst I digests of Thermoplasma

DNA were electrophoretically separated in 2% agarose gels. The separated DNA fragments were transferred onto nitrocellulose by Southern blotting, and hybridized with nick-translated rDNA plasmids. The hybridization result is shown in Fig. 18; the sizes of the hybridized bands are summarized in Table 5. In addition to the known rRNA hybridized bands listed in Table 1, some new bands appear (see Table 5, marked with stars (★)). These represent restriction fragments which flank the cloned DNA sequences. They do not hybridize with 23S and 16S rRNA, but with the cloned rDNA fragments. By relating the sizes of the hybridized bands to the known Bgl I, Cla I, Eco RI and Pst I restriction sites in the cloned fragments (Fig. 12), the next sites for these enzymes to either side of the whole cloned region have been determined (Fig. 19).

Table 5. Sizes (Kb) of Thermoplasma DNA restriction fragments which hybridize to nick-translated plasmids

enzyme used for digesting Thermoplasma DNA	nick-translated plasmid used for hybridization			
	pTB52	pTH1101	pRB224	pTH126
Bgl I	3.7, 1.1★	12.4, 3.7	12.4	2.9
Cla I	6.6, 2.42★	6.6	6.6	5.5, 0.67★
Eco RI	9.7	9.7	9.7, 2.12★	5.3
Pst I	3.7, 3.3	3.7, 3.3	3.3, 3.0★	9.25

★ denotes those fragments which are not found in the rRNA-DNA hybridization (see Table 1)

Fig. 19 shows the detected 16S rRNA-gene containing DNA

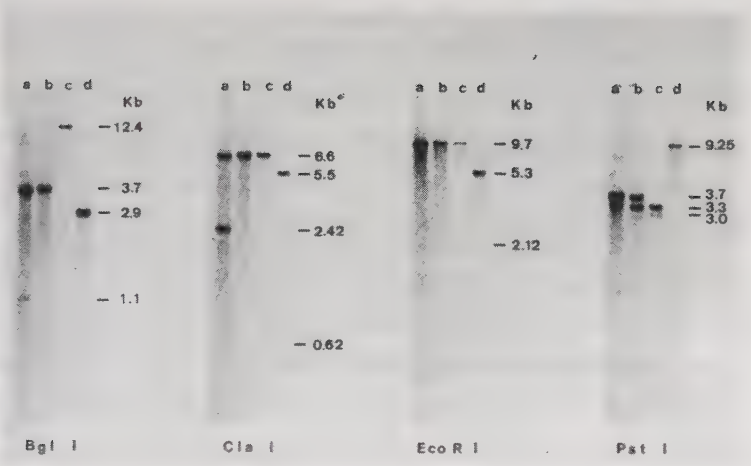

Fig. 18 Hybridization of restriction endonuclease digested Thermoplasma DNA with the nick-translated plasmids carrying Thermoplasma rDNA.

Thermoplasma DNA was digested separately with Bgl I, Cla I, Eco RI and Pst I. Fragments were electrophoretically separated in 2% agarose gels and then transferred onto nitrocellulose sheets. The plasmids pTB52 (track a), pTH1101 (track b), pRB224 (track c) and pTH126 (track d) were nick-translated and hybridized to the nitrocellulose sheets. Hind III digested T5 phage DNA fragments were used as size standards.

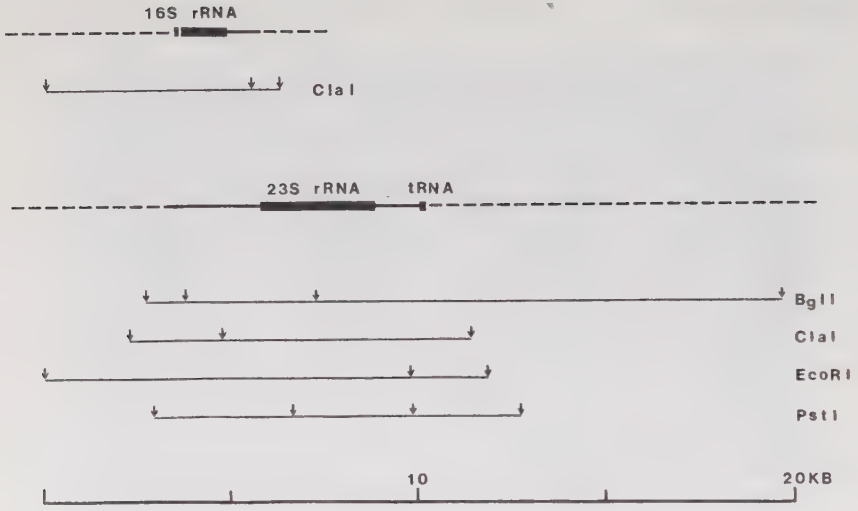

Fig. 19 Schematic representation of the detected fragments flanking the cloned Thermoplasma rDNA-containing regions.

The cloned Thermoplasma rDNA-containing regions are represented by continuous lines. The fragments flanking the cloned regions which are detected by hybridizing restriction endonuclease digested Thermoplasma DNA with the nick-translated plasmids, are presented by dashes. The positions of 23S rRNA gene, 16S rRNA gene, and tRNA gene(s) are represented by the bars.

sequence of about 6.3 Kb in size, with the next Cla I sites at the ends, in the Thermoplasma genome. It contains two DNA fragments about 3.4 (left) and 1.5 Kb (right) in size respectively, to either side of the 16S rRNA gene. The detected 23S rRNA-gene containing DNA sequence of about 20 Kb between an Eco RI site (left) and a Bgl I site (right) contain DNA fragments of about 6 (left) and 11 Kb in size (right) respectively on the sides of the 23S rRNA gene. In all hybridizations, no common band between the plasmid carrying 16S rDNA and the plasmids carrying 23S rDNA has been discovered. This means the distance between 16S rRNA gene and 23S rRNA gene in Thermoplasma genome should be at least larger than the sum of the sizes of the shorter DNA fragments flanking the rRNA genes in Fig. 19, i.e. 7.5 Kb (1.5 + 6 Kb).

By comparing the hybridization patterns in Fig. 18 and the sizes of the hybridized fragments in Table 5 with those in Fig. 8 and Table 1, no linkage relationships (common hybridized bands) between 5S and 23S or 5S and 16S rRNA genes have been found. This means that if all six enzymes cut Thermoplasma DNA just beside the 5S rRNA gene, the distances between 5S and 23S, and 5S and 16S rRNA genes would still be at least 6 and 1.5 Kb respectively.

4. DISCUSSION

4.1 THERMOPLASMA RIBOSOMES AND rRNAs

4.1.1 RIBOSOMES

Like eubacteria and chloroplasts, Thermoplasma contains ribosomes belonging to the 70S class, with an S-value of 64S as estimated by sucrose gradient centrifugation with E. coli ribosomes as standard. They dissociate into 45S and 32S subunits.

Mg^{++} ions are required for the stabilization of the ribosomal structure. The peaks of ribosomes and ribosomal subunits disappeared when sucrose gradient centrifugation was performed in the presence of 1 mM EDTA and in the absence of Mg^{++} in the buffer. Bacterial, chloroplast, and mitochondrial ribosomes reqire at least 5 mM Mg^{++} for stability (44,98,106), and dissociate into 50S and 30S subunits if the Mg^{++} concentration is lowered to 1mM. In contrast, eukaryotic ribosomes are relatively stable at low Mg^{++} concentrations (30). In the range of 10-100 mM Mg^{++}, no apparent 64S peak of Thermoplasma ribosomes was visible at 260 nm in addition to the subunit peaks (45S and 32S). When Thermoplasma cells were labeled with ^{32}P for 20 min, however, a 64S ribosome peak could be clearly recognized (Fig. 2 and 3). Therefore, Thermoplasma ribosomes are very

unstable in the absence of mRNA, dissociating into their subunits, i.e. the affinity between the two ribosomal subunits is much lower than those of bacteria, chloroplasts, mitochondria or eukaryotes.

The small ribosomal subunit of Thermoplasma has a function comparable to that of other organisms in mRNA binding. The binding of mRNA to 30S ribosomal subunits is the first step in translation initiation. Shine and Dalgarno (102) described the existence of a purine-rich stretch 7-15 bases upstream from the initiation triplet AUG on mRNAs in E. coli complementary to 3-7 bases in a pyrimidine-rich stretch near the 3'-end of the 16S rRNA. Whether there is a Shine-Dalgarno sequence-like structure on Thermoplasma mRNA for translation initiation or whether the 3'-terminal of Thermoplasma 16S rRNA, which has not yet been sequenced, contains the complementary sequence, is not yet clear.

4.1.2 rRNAs

Thermoplasma has three stable rRNAs in ribosomes, a 16S rRNA (0.48×10^6 d.) in the 32S subunit, a 23S rRNA (1.2×10^6 d.) and a 5S rRNA in the 45S subunit, and in addition a second 23S rRNA which is presumed to be a gel artifact.

rRNA processing has been very intensively studied in E. coli and some other prokaryotes and eukaryotes . In general, the rRNAs have a common precursor (except the 5S rRNA in eukaryotes and the rRNAs in the mitochondria of lower eukaryotes) which is then processed by special RNases into

stable rRNAs and some tRNAs. The accumulation of 30S p-rRNA in chloramphenicol treated E. coli has been observed (101). This 30S precursor including its 5'-triphosphate has been isolated from an RNase III$^-$ strain (5,26,36,71). Ghora and Apirion showed the release of tRNA and 5S rRNA by treating this 30S p-rRNA with RNase P and 'E' (35). Similar phenomena have been found in mammals (67), Bombyx mori (88), lower eukaryotes (89), and perhaps spinach chloroplasts (49). In Thermoplasma, common precursors of 5S, 16S and 23S rRNA with a high molecular weight have not been found. There are some ^{32}P-labeled high molecular weight bands above the 23S rRNA in cell lysates (Fig. 3 and 4). They were eluted from gels, treated with DNase, RNase A and nuclease S1, then analysed by gel electrophoresis. They are sensitive to all of those nucleases, and no apparent RNA bands have been seen. Thus, they could be complexes of DNA and RNAs.

The smaller stable 23S rRNA (molecular weight of 1.1×10^6 d.) may be an artifact of the larger one. The reasons for this conclusion are 1. Both of the 23S rRNA bands are non-chaseable, but they hybridize to the same restriction fragments of Thermoplasma DNA; no heterogeneity of the 23S rRNA gene has been found (see Fig. 6). Therefore, they must be transcribed from the same 23S rRNA gene; neither represents a precursor of the other one. 2. The hybridization of 23S rRNA to the Taq I-restricted rDNA-carrying plasmids showed that there is a DNA sequence of 340 bp in plasmids pTB82, pTB52 and pTH1101 which is strongly hybridized by 23S rRNA in some experiments but not in

others, no matter which of the two 23S rRNAs was used. This can be explained as an artifact caused by the secondary structure of a short sequence in 23S rRNA (for example hairpin) in this region, rather than by the assumption that it is due to an intervening sequence in 23S rRNA. This secondary structure can change the apparent size of the RNA such that it appears about 300 nucleotides (ca. 0.1×10^6 d) shorter.

The 16S rRNA may have two precursors, the rRNAs of molecular weights of 0.59×10^6 and 0.51×10^6, because they are chaseable and hybridize to the same DNA fragments as the 16S rRNA. No apparent artifact has been found in 16S rRNA.

4.2 CLONING AND RESTRICTION MAPPING OF THE CLONED THERMOPLASMA rDNA

4.2.1 CLONING

Five different clones, three from the Bam HI batch and two from the Hind III batch, have been isolated after analysing more than 40 clones which hybridized with ^{32}P-labeled rRNAs. Four of these clones (containing the pTB82, pTB52, pTH1101 and pRB224) contain partial fragments of the 23S rRNA gene. The cloned rDNA fragments in those plasmids can be reconstituted to a sequence containing an entire 23S rRNA gene, and two rRNA-nonhybridizing flanking fragments which are about 1.2 and 2.6 Kb long respectively. The fifth contains pTH126 carrying a part of the 16S rRNA

gene (1.2 Kb), and an rRNA-nonhybridizing DNA fragment of about 0.9 Kb. The remainder of the 16S rRNA gene and the whole 5S rRNA gene have not been isolated.

The fact that these two parts of rDNA are missing can be explained by two reasons: 1. In colony or restricted DNA fragment hybridization, the hybridizing capacity (both with or without subsequent RNase digestion) depends very strongly on the length of the stretch able to hybridize. The missing 5S rDNA clones in both batches and that of the 16S rDNA in the Hind III batch have a length of only about 100-200 bp to hybridize and thus could easily fail to be detected in the presence of weak background. 2. The vector used in this work is pBR322. It is well known that in cloning with plasmid vectors the chance of isolating clones decreases with increasing size of the fragment to be cloned. The missing 16S rDNA clone in the Bam HI batch should contain an insert of about 6.7 Kb. In shotgun cloning, fragments of such size are generally recessive. The choice of Bam HI and Hind III was made on the basis of the small average sizes of the hybridized DNA fragments (see Table 1). The special rDNA organization found in Thermoplasma was unexpected; otherwise cloning could have been performed using cosmids or Charon lambda.

4.2.2 RESTRICTION MAPPING

The plasmids carrying Thermoplasma rDNA have been physically mapped by Bam HI, Bgl I, Cla I, Eco RI, Hind III, Pst I, Taq I and Hinf I restrictions. Except those of Hinf

I, all the restriction data are discussed in RESULTS. The overlap of the inserts of the pTB82, pTB52, pTH1101 and pRB224 is consistent with the restriction pattern and the hybridization data. There is a common Pst I site in the inserts of pTB82, pTB52 and pTH1101; and another Pst I site on the far side of the insert of the pRB224 (Fig. 12). These two Pst I sites are about 3.2 Kb apart. Two Bgl I sites on the whole region covered by the four cloned fragments are separated by about 3.5 Kb. There is an Eco RI site on one side of this region, and a Cla I site on the other side. Of these enzymes only Cla I cuts the insert of the pTH126, which contains part of the 16S rRNA gene (Fig. 12).

Taq I sites have been recognized by comparing the restriction patterns of the pBR322 vector and the plasmids digested by 1. Taq I, 2. Taq I + Bam HI 3. Taq I + Hind III, and 4. Taq I + Bam HI + Hind III. The Taq I sites neighboring both ends of inserted fragments, a Bam HI site or a Hind III site can be directly identified. However, the Taq I sites in a long DNA sequence, not dealing with any end or the sites of those two enzymes, can be detected by hybridization, if a rRNA-hybridized fragment lies in the stretch between this Taq I site and another known site. Naturally, the prerequisite for this kind of recognition is the absence of intervening sequences in rDNA. This seems to be the case because all Taq I sites identified in this way are located in the terminal sequences of the rRNA genes. So far, the cloned rDNA-rRNA hybrids have not yet been analysed by electron microscopy for the occurrence of R-loops, a good

criterion for intervening sequences. The precise positions of some Taq I sites between rRNA genes and insertion points have not been identified; in these cases the sizes (bp) of the corresponding restriction fragments are given below the stretch between the two neighboring known Taq I sites. One of the Taq I sites in pTH126, the plasmid carrying 16S rDNA, has been recognized as a Sal I site (Fig. 15). There is no Sal I site in the cloned 23S rDNA fragments.

The cloned Thermoplasma rDNA fragments were also mapped with the restriction enzyme Hinf I. On one side of the reconstituted 23S rRNA-gene containing DNA sequence (covered by the inserts of pTB52 and pTH1101) Hinf I digestion gives fragments of suitable sizes for mapping. But on the other side of this region (covered by the insert of the pRB224) and the insert of the 16S rDNA-carrying pTH126 Hinf I restricts the DNA into many small fragments of 50-100 bp (data not shown), making mapping impossible. Eventually this could be an important source of information about the DNA structure outside the rRNA genes. Because rRNAs and tRNAs do not hybridize to these small fragments, it is reasonable to assume that there are -GANTC- (Hinf I recognition sequence) repeated sequences before or after both 16S and 23S rRNA genes.

4.3 THERMOPLASMA rDNA STRUCTURE

The result of hybridizing rRNAs to restricted Thermoplasma DNA shows that the genes for the three rRNAs

could be far apart. Since no information about the sizes of spacers had been obtained, the Thermoplasma rDNA was cloned. After restriction mapping and hybridization, it became clear that the cloned fragments represented two independent groups, i.e. an entire reconstituted 23S rRNA gene with two flanking fragments (1.2 and 2.6 Kb respectively) and an incomplete 16S rRNA gene (1.2 Kb) with a rRNA-nonhybridized DNA fragment (0.9 Kb). There is no linkage relationship between these two groups. No cross hybridization between 16S rRNA and the plasmids carrying 23S rDNA and vice versa has been found. This means that the spacer between 16S rRNA gene and 23S rRNA gene is at least 1.2 Kb (the size of the shorter DNA fragment beside 23S rRNA gene). To determine the maximal detectable distances between rRNA genes, nick-translated plasmids were used. The idea was that the cloned rDNA fragments cover much larger DNA sequence than the rRNA genes themselves. By relating the sizes of plasmid-hybridizing DNA fragments to the known restriction sites in the cloned fragments, the distance from known sites in the whole cloned region to the next site outside it could be measured.

The analysis showed that Thermoplasma has a very special rDNA organization which has never been reported in any organism. Linkages between 23S, 16S and 5S rRNA genes have not even been found in the regions between the two known furthest restriction sites in the DNA fragments flanking the whole cloned DNA sequences (ca. 20 and 6.3 Kb) (Fig. 19). Instead of forming a gene cluster, they thus seem to be

distributed separately in the genome. The spacers between 16S and 23S, 23S and 5S, and 5S and 16S rRNA genes are at least 7.5, 6, and 1.5 Kb respectively. Such large spacers have only been reported between the genes coding for the 'large rRNA' and the 'small rRNA' in mitochondrial genome of primitive eukaryotes, e.g. yeast (31, 100) and Neurospora (52,74) which, however, do not posses 5S rRNA.

4.4 TRANSCRIPTIONAL CONTROL OF rRNA GENES IN THERMOPLASMA

It is questionable that there is only one promoter for all three rRNA genes in an organism which has a rDNA organization like Thermoplasma. A comparable case is the rDNA organization in Neurospora mitochondria, with a spacer of about 5 Kb between 19S rRNA gene and 25S rRNA gene (52,74). Separate precursors for 19S rRNA and 25S rRNA have been clearly demonstrated by Green et al. (42,43), i.e. the two rRNA genes are transcribed from two independent transcription units. The spacers between Thermoplasma rRNA genes are larger than that between the two Neurospora mitochondrial rRNA genes (with the possible exception of the spacer between 16S and 5S rRNA genes, which must still be at least 1.5 Kb). No common transcript containing more than one rRNA has be found in ^{32}P-pulse labeled Thermoplasma cells. The two detected p-rRNAs are precursors of 16S rRNA, and do not hybridize to 5S rRNA-gene containing DNA fragments. Thus the three Thermoplasma rRNA genes very probably constitute three separate transcription units.

It has been well established that the ribosomes of *E. coli* consist of three rRNAs and about fifty proteins. The synthesis of rRNAs and ribosomal proteins has been reported to show a coordinate stringent response upon amino acid starvation (22). The three separate rRNA transcription units in the *Thermoplasma* genome should also be coordinately controlled to ensure a constant ratio between the rRNAs. This should be a suitable system for studying coordination between several genes belonging to the same class and it offers some advantages for studying the process of transcriptional control. The gene products are simply three rRNAs which can be easily detected. *Thermoplasma* is a free-living cell which grows in a very simple medium so that the experimental conditions can be easily controlled. It has a prokaryotic chromosome without a nuclear membrane and lacks a rigid cell wall and the genes can be easily manipulated.

4.5 WHAT IS THE 'TYPICAL ARCHAEBACTERIAL rDNA ORGANIZATION' ?

Since the establishment of the concept of archaebacteria *Halobacterium* has been the most studied organsiam. The rDNA organization of *Halobacterium halobium* has been described by Hofman et al. (54). One set of rDNA with an organization similar to that found in eubacteria, of the 5'-16S-23S-5S-3' type, was reported. No apparent precursor for 16S and 23S rRNA was found. In this thesis, a completely different type

of rDNA organization has been demonstrated for Thermoplasma. So far these are the only cases where archaebacterial rRNA gene organization has been studied. However the experimental technique on which the claim of a eubacterial rDNA organization in Halobacterium rests is questionable. That the 16S rRNA used in those experiments is contaminated by 23S rRNA can be clearly seen from the results of the gene copy number determination for the 16S and 23S rRNA genes. The existence of only one set of 23S rRNA gene has been shown in the saturation curve by adding an excess of 23S rRNA. The saturation level with 16S rRNA is about 50% higher than that with 23S rRNA implying three copies of 16S rRNA gene. However, this level of hybridization is also achieved by using a mixture of 16S and 23S rRNA. Since the size ratio of 23S to 16S rRNA is approximately 2:1, it is probable that only one copy of each gene exists. The same phenomenon has also been observed in the experiments reported here. Hybridizing the contaminated 16S rRNA to restricted chromosomal DNA without adding an excess of cold 23S rRNA may sometimes lead to wrong conclusions. Fragments may sometimes hybridize strongly with the contaminating 23S RNA breakdown products whereas the correct fragments may hybridize weakly with the 16S rRNA itself (see Fig. 20). The same situation has also been observed for the isolated 5S rRNA. The work reported here was hampered by this problem for a long time. It is important to point out that the artifactual bands do not necessarily show lower hybridization capacities than the correct ones. Hybridization depends

strongly on the size of the hybridizable DNA fragment, the secondary structures of the DNA and the RNA and the transfer efficiency of the sheet blotting etc.. When the contaminating 23S rRNA becomes notable in the saturation curve of the contaminated 16S rRNA, the impurity is high enough to be carefully controlled. The question remains whether Thermoplasma rDNA organization represents the archaebacterial type of rDNA organization. This question will be answered by studying known taxonomic orders of archaebacteria including Halobacterium. In addition cloned Thermoplasma rRNA gene promoter(s) shall be detected, sequenced and compared with eubacterial and eukaryotic rDNA promoters. The evolutionary relationship between Thermoplasma rDNA and mitochondrial rDNA should also be checked.

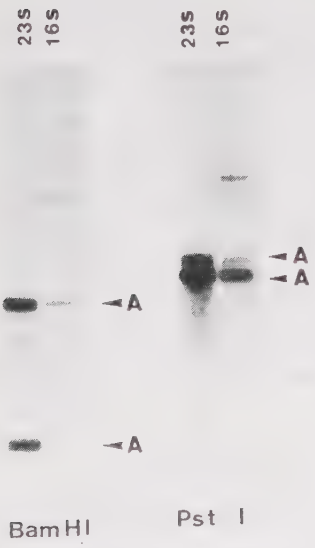

Fig. 20 Hybridization by using contaminated rRNA.

Bam HI and Pst I digested Thermoplasma DNA fragment were hybridized with 23S and 16S rRNAs without adding contaminating rRNA (in this case 23S) for quenching. The bands which are resulted by contamination are marked by A.

5. MATERIALS

5.1 ORGANISMS

Thermoplasma acidophilum, 122-1B2 obtained from E. A. Freundt, Aarhus, Denmark.

E. coli C600

5.2 CHEMICALS AND RADIOISOTOPES

5.2.1 CHEMICALS

Unless otherwise specified, all chemicals are reagent grade, purchased from E. Merk, Darmstadt, FRG

Acrylamide	Serva, Heidelberg, FRG
Agarose	SeaKem, Marine Colloids, Rockland, USA
Bacto-agar,-tryptone	Difco Labs., Detroit, USA
Calf thymus DNA	Sigma, St. Louis, USA
Nitrocellulose, BA 85, 0.45 µm	Schleicher and Schuell, Dassel, FRG
N,N'-methylene bisacrylamide	Serva, Heidelberg, FRG
NPBC (nitrobenzyl-oxymethyl-pyridinium-chloride)	BDH, Poole, UK
Urea (ultra pure)	BRL, Gaitherburg,
Yeast extract	Difco Labs., Detroit, USA
Yeast RNA	Boehringer, Mannheim, FRG

5.2.2 RADIOISOTOPES

All radioisotopes are purchased from Amersham Buchler, Frankfurt, FRG

Adenosine 5'-(γ-^{32}P) triphosphate, 3000 Ci/mmol.

Deoxycytidine 5'-(α-^{32}P) triphosphate, 400 Ci/mmol.

^{32}P-orthophosphate, carrier free

5.3 BIOCHEMICALS AND ANTIBIOTICS

5.3.1 ANTIBIOTICS

Ampicillin	Sigma, St. Louis, USA
Chloramphenicol	Serva, Heidelberg, FRG
Tetracycline	Serva, Heidelberg, FRG

5.3.2 ENZYMES

Calf alkaline phosphatase	Boehringer, Mannheim, FRG
DNA endonucleases (restriction enzymes)	Boehringer, Mannheim, FRG
DNase I (RNase free)	Worthington, Freehold, USA
DNA polymerase (Kornberg enzyme)	Boehringer, Mannheim, FRG
Lysozyme	Sigma, St. Louis, USA
Polynucleotide kinase	Boehringer, Mammheim, FRG
RNase A, and T1	Boehringer, Mannheim, FRG
T4-ligase	NEN, Dreieich, FRG

5.4 MEDIA AND BUFFERS

5.4.1 MEDIA

Thermoplasma medium	ml/100 ml of medium
10% Thermobasa	10
20% Glucose	5
10% LP-yeast extract	1

10x Thermobasal	g/l
$(NH_4)_2SO_4$	13.2
K_2SO_4	2.38
$MgSO_4 \cdot 7H_2O$	2.47
$CaCl_2 \cdot H_2O$	0.74

adjusted to pH 0.2 with 9 M H_2SO_4, then autoclaved

10% LP (low-phosphate) yeast extract solution

The low-phosphate yeast extract solution was prepared by a modified method described originally by Pinkerton et al. for the preparation of low-phosphate peptone (91). 80 ml of 0.25 M $MgSO_4$ was added to 100 ml of 20% Difco-yeast extract solution. The pH was brought to 10.5 by adding 10 M KOH. After removing the precipitate by centrifugation, the clear supernatant was adjusted to pH 6.3 with 9 M H_2SO_4 and then autoclaved for 20 min.

LB Medium	g/l
Difco Bacto-tryptone	10
NaCl	10
Difco yeast extract	5

LB agar

1.5% Difco agar in LB medium

In LB agar-antibiotic plates the antibiotic ampicillin, tetracycline and chloramphenicol were given to the concentration of 50 µg, 25 µg and 200 µg/ml respectively.

5.4.2 BUFFERS

10x TEN buffer

Tris/HCl	0.2 M	pH 7.6
NaCl	0.2 M	
Na_2EDTA	0.01M	

10x Loening buffer (66)

Tris	0.36M	
NaH_2PO_4	0.3 M	
Na_2EDTA	0.01M	
		pH 7.8

10x Sample buffer

Bromophenol blue	20 mg/ml
Orange G	20 mg/ml
Xylenecynol	20 mg/ml
Sucrose	50%
Na_2EDTA	10 mM

10x TBE buffer

Tris	0.89 M	
Boric acid	0.89 M	
Na_2EDTA	0.024M	
		pH 8.3

TU buffers

10x TU A

Citric acid	0.25M

β-alanine	0.5 M	
		pH 3.6

10x TU B

Citric acid	0.25M	

20x SET

Tris/HCl	1 M	pH 8.0
NaCl	3 M	
Na_2EDTA	20 mM	

20x SSC

NaCl	3.0 M	
$Na_3citrate$	0.3 M	
		pH 8.1-8.3

Hybridization buffer

Formamide	50%
SET	5x
SDS	0.1%
Yeast RNA (or sonicated, heat-denatured DNA)	10 mg/ml

Elution buffer (for RNA and DNA form gels)

Elution buffer A

1x TBE

Elution buffer B

Glycerol	75%
Na-acetate	0.5 M
TBE	1x

TEMN buffer

Tris/HCl	20 mM	pH 7.8
$MgCl_2$	25 mM	

NaCl	40 mM
Na_2EDTA	0.1mM

Phenol reagent

Phenol	100 ml
1M Tris/HCl pH 8.0	100 ml
8-hydroxyquinoline	100 mg

6. METHODS

6.1 GELS FOR RNA

6.1.1 Acid-urea agarose gel

The acid-urea agarose gel (99) was modified to analyse the total RNAs and prepare the large rRNAs.

For every 10 ml of gel, 0.2 g of agarose was heated in 2.23 ml of water in a autoclave for 5 min. 6.67 ml of pre-warmed (50°C) 9 M urea solution was added to the viscous agarose solution in a 70°C water bath. The mixture was quickly cooled to room temperature after good mixing at 70°C for 30 sec. 0.75 ml of TU B, 0.25 ml of TU A and 0.1 ml of 10% SDS were added and mixed. After pouring the material in a suitable gel former, the gel was kept at 4°C for at least 4 hr.

The samples were prepared in the following way: 10 µl of probe and 2 µl of 10x sample buffer were added to 7.5 mg of urea, vortexed, then heated at 70°C for 2-3 min. After

chilling the mixture on ice, 2 µl of acidic solution (TU A/TU B, 1/4) were added.

Electrophoresis was carried out for 16 hr at 5V/cm of gel and 4°C. The running buffer was 10-fold diluted TU A containing 0.1% SDS.

6.1.2 Polyacrylamide gel

10% polyacrylamide (acrylamide/bis = 9.6/0.4) gel containing 6 M urea in TBE buffer was used for preparation of 5S rRNA and tRNA.

6.2 GELS FOR DNA

6.2.1 Agarose gel

Horizontal agarose gel slabs (78) were used for analysis and preparation of large DNA fragments.

Hind III digested T5 phage DNA fragments were used as size standards.

6.2.2 Polyacrylamide-agarose composite gel

To analyse the small DNA fragments (20-2000 bp), the polyacrylamide-agarose composite gel described originally by Peacock and Dingman (85) was modified. Instead of TBE buffer, Loening buffer was used in order to avoid the running front.

Hae III and/or Alu I digested pBR322 DNA fragments were

used as size standards.

6.3 RNA AND DNA ELUTION SYSTEM

The glycerol gradient electrophoresis (41) method was modified to elute the RNA and DNA fragments from the gels.

5 steps (elution buffer A/B = 4/0, 3/1, 2/2, 1/3 and 0/4) of gradient were established in a 3-ml glass tube (diameter 6 mm) whose bottom was sealed with a piece of celluphane membrane by an O-ring. On the upper side of the glass tube, a disposable 30-ml syringe was fixed. The cut gel piece was put into the syringe, and eluted by electrophoresis.

After overnight elution (normally 40-60V at room temperature), the gradient was fractionated in 0.3 ml samples. For hot probes the fractions were counted by Cerenkov counts. Cold samples were detected under UV by injecting 1-5 µl of probe with an Eppendorf pipette into a 0.1% agarose plate containing 1 µg/ml of ethidium bromide.

6.4 PREPARATION AND ANALYSIS OF THERMOPLASMA RIBOSOMES

The buffer used in all procedures for Thermoplasma ribosome preparation was TEMN buffer. Crude Thermoplasma ribosomes were prepared by centrifuging Triton-lysed Thermoplasma cells. The centrifugation was carried out in a Beckman 60-Ti rotor at 50,000 rpm and 15^{o}C for 6 hr.

The pellet was dissolved in buffer by shaking overnight

at 4°C for further purification and analysis.

6.4.1 SW-41

The ribosomes were analysed and prepared (small amounts) by centrifuging in a 10-30% sucrose gradient in the Beckman SW-41 rotor. The centrifugation was carried out for 150-180 min at 35,000 rpm and 15°C.

6.4.2 Zonal rotor

For large scale preparation of Thermoplasma ribosomes, the Beckman Ti-16 zonal rotor was used. A logarithmic sucrose gradient was built up with 230 ml of 10% sucrose and 700 ml of 40% sucrose. 20 ml of crude ribosome preparation in 5% sucrose was applied on the gradient, then overlayered with 40 ml of buffer, The centrifugation was carried out for 5 hr at 45,000 rpm and 15°C.

6.5 PREPARATION OF rRNAs

The chloroform-phenol-SDS method (84,90) was used for preparation of RNAs from ribosomes.

The ribosomes were phenolized by adding 1/20 vol. of 10% SDS and 1 vol. of phenol reagent. After adding another vol. of chloroform, the mixture was shaken vigorously for a few seconds, shaken again 2-3 min later, and then centrifuged for 3 min. The phenol-chloroform phase was drawn out carefully with a Pasteur pipette. The water phase and interphase were re-extracted twice with 2 vol. of

chloroform. 16S and 23S rRNA were precipitated with 3 M Na-acetate by adding 5 M Na-acetate to the water phase (83). The 5S rRNA was recovered from the 3 M Na-acetate supernatant by centrifugation after the addition of 2 vol. of absolute ethanol.

The RNAs were further purified by gel electrophoresis when necessary.

6.6 PREPARATION OF HOT rRNA PROBES

The hot rRNA probes in all experiment were prepared either by 5'-terminal labeling *in vitro* or by growing the cells in $H_3{}^{32}PO_4$ containing *Thermoplasma* medium *in vivo*.

6.6.1 *In vivo*

An LP-culture in late log phase (normally, A_{540} = 0.4) was diluted 200-fold into fresh LP-medium. After 2 hr growth, carrier free $H_3{}^{32}PO_4$ was given to a final activity of 25 (or 100) µCi/ml. The *Thermoplasma* cells were harvested 3 days later.

The harvested cells were washed twice with 50 mM acetate buffer pH 5.0, then lysed by adding TEMN buffer and triton (0.01%). The lysate was centrifuged in a Beckman air-fuge for 3 hr. *Thermoplasma* tRNAs were precipitated from the supernatant with ethanol after phenolization. The rRNAs were prepared from the pellet by the procedures described above (in 5.5 PREPARATION OF rRNAs).

6.6.2 *In vitro*

The RNAs were dephosphorylated by calf alkaline phosphatase, and labeled with γ-^{32}P-ATP by polynucleotide kinase (13).

0.5 U of calf alkaline phosphatase, 4 U of polynucleotide kinase and 500 µCi (150 pmol) of γ-^{32}P-ATP were used for labeling 10 µg, 5 µg, 1 µg and 1 µg of 23S, 16S, 5S rRNA and tRNAs respectively.

6.7 PREPARATION OF DNAs

6.7.1 *Thermoplasma* DNA

The *Thermoplasma* DNA was preparated from the clear cell lysate by phenolzation, and then banded twice by equilibrium CsCl centrifugation.

6.7.2 Plasmid DNAs

The plasmid DNA preparation procedure described by William et al. (50) was used in this thesis.

The plasmid-containing *E. coli* C600 strains were grown in LB medium. The plasmids were amplified by treating the cells with chloramphenicol for 18 hr. The harvested, washed cells were lysed by treatment with lysozyme and EDTA, followed by Triton. After two equilibrium CsCl centrifugations the supercoiled plasmid DNAs were extracted with isopropanol until ethidium bromide free, then dialysed against TEN buffer.

6.8 NICK-TRANSLATION

Nick-translation was used for labeling the DNAs *in vitro* (95).

1 µg of DNA and 50 µCi of α-^{32}P-dCTP (120 pmol) were catalysed by 10 U of DNA polymerase in 100 µl of mixture contaning 20 mM Tris/HCl pH 7.5, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, 200 µg/ml of BSA and 1 µM of each cold dATP, dGTP, dTTP. After 2 hr incubation at 14°C, the nick-translated DNAs were separated from the nonreacted nucleotides by a sephadex G50 column (0.7 cm x 20 cm) in TEN buffer. The DNA peaks were pooled, denatured by adding of NaOH to 0.5 M (in ice for 1 hr), and used for hybridization immediately after neutralization.

6.9 TRANSFER OF DNA ONTO NITROCELLULOSE OR DBM PAPER

6.9.1 Southern blotting

DNA fragments larger than 200 bp were transferred onto nitrocellulose, using essentially the method of Southern (103).

Gels containing DNA bands were submersed in 0.5 M NaOH-1.5 M NaCl for 30 min at room temperature. After neutralization twice in 1 M Tris pH 7.4-0.6 M NaCl for 30 min at room temperature, the gels were equilibrated for 10 min with 20xSSC and then transferred to the nitrocellulose

sheets which had been pre-soaked with 20xSSC.

6.9.2 Transfer of DNA to DBM paper

DBM (diazobenzyloxymethyl) paper was mainly used for hybridization experiments. It was prepared according to the method of Goldberg et al. (40).

DNA fragments in the gel were partially depurinated by shaking the gel twice in 0.25 M HCl for 15 min at room temperature. After rinsing in distilled water, the gel was treated twice with 0.5 M NaOH-1.0 M NaCl for 15 min and equilibrated twice in 1 M Na-acetate pH 4.0 for 20 min at room temperature. DNA fragments were then transferred from the gel to a activated DBM sheet by the method of Southern, but in the cold room (1)

6.10 SHOTGUN CLONING AND COLONY HYBRIDIZATION

6.10.1 DNA digestion and ligation

Thermoplasma DNA and pBR322 were digested (with Bam HI or Hind III) separately. After phenolization the DNAs were precipitated with ethanol.

2 µg each of digested Thermoplasma DNA and pBR322 were ligated by 0.5 U of T4-ligase in 20 mM Tris/HCl pH 7.8, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, 1 mM ATP and 200 µg/ml of BSA at 14°C for 16 hr (25).

6.10.2 Transformation

40 ml of freshly inoculated C600 culture was grown to A_{650} of 0.2. The culture was chilled for 10 min on ice, then harvested by centrifugation. The cells were resuspended in 20 ml of cold (0°C) 0.1 M $CaCl_2$. After calcium shock at 0°C for 30 min, the cells were spun down by centrifugation and resuspended in 0.5 ml of cold $CaCl_2$ (19).

20 µl of ligated DNA (2 µg of Thermoplasma/2 µg of pBR322) was added to 200 µl of calcium-treated C600 suspension. The mixture was kept in ice for 30 min, heated at 42°C for 2 min, then chilled in ice for 15 min.

The transferred cells were incubated at 37°C for 45 min after addition of 0.8 ml of LB medium. Before plating the cells were spun down by centrifugation and resuspended in 0.2 ml of LB medium.

6.10.3 Colony hybridization and high density plasmid screening

The procedure described below was modified in order to save work and increase the reliability of the method described originally by Grunstein and Hogness (45).

0.2 ml of transferred cell suspension which can form around 5,000 ampicillin resistent colonies was spread onto a sterile nitrocellulose sheet (11x11 cm^2) on an LB-agar plate containing 50 µg/ml of ampicillin. The plate was then incubated at 24°C. When the colonies reached 0.1 mm, a

replica was made by pressing a moist sterile nitrocellulose sheet face-to-face with the template together on a piece of velvet. The template and replica were incubated on fresh ampicillin-LB plates at 37°C until the colonies reached about 0.5 mm.

The template was kept in the cold room, whereas the replica was transferred to a chloramphenicol containing LB plate and incubated at 37°C for 20 hr. After incubation the replica was laid twice on 2.5 ml of 0.5 M NaOH for 2-3 min, and dried briefly on paper towels between every step, then neutralized twice with 2.5 ml of 10xSET, air-dried on paper towels. Before baking 3 hr at 70°C in vacuum, the sheet was rinsed with 0.3 M NaCl and air dried. The sheet was then used for hybridization with labeled rRNAs.

After hybridizing to ^{32}P-labeled Thermoplasma rRNAs, the nitrocellulose sheet was washed twice with 2xSET, treated with RNase A until the radioactivity in the digests reached a minimal plateau. The sheet was then washed with 2xSET-0.1% SDS. An autoradiograph was made from the sheet at -70°C.

The regions of hybridized spots were picked up, and selected once more by the described procedure. But instead of around 5,000 colonies, 200 colonies were applied on a sheet. Single hybridized colonies were isolated from the selection sheets.

6.11 HYBRIDIZATION CONDITIONS FOR SOUTHERN SHEETS AND DBM PAPERS

All hybridizations were done by sealing the hybridization set (nitrocellulose sheet and hot probes in hybridization buffer) in a plastic bag, and incubated at 42°C overnight.

6.12 MINI-LYSATE

For the detection of the molecular weight of a inserted fragment in the plasmid, a time- and material-saving method (mini-lysate) was used.

Cells containing plasmid in 1 ml of overnight culture without chloramphenicol treatment were harvested in an Eppendorf centrifuge and resuspended in 100 µl of 50 mM Tris pH 7.5, 1 mM EDTA. After adding 10 µl of 10% SDS and incubating the mixture for more than 15 min at room temperature, a clear cell lysate was recovered by centrifugation. The clear cell lysate was then phenolized and precipitated with ethanol. The precipitate was resuspended in 20 µl of RNase A solution (10 µg/ml of the same buffer described above) and incubated for 30 min at 37°C. Restrictions were done by adding 1/10 vol. of 10x restriction buffer and the restriction enzyme, after RNase A digestion.

6.13 DETERMINATION OF GENE COPY NUMBER

Techniques of the determination of gene copy number were carried out mainly according to De Ley and De Smedt (21).

6.13.1 Fixation of denatured DNA to membrane filters

1.2-1.5 mg of Thermoplasma DNA was dissolved in 50 ml of 6xSSC. After adding 5 ml of 5 M NaOH to the DNA solution, the mixture was incubated in ice for at least 1 hr to denature the DNA. The denatured DNA was reneutralized by titrating with 6 M HCl, and 10 ml of this solution (contains about 200 µg of DNA) was then filtrated immediately through a nitrocellulose membrane filter (diameter 2.4 cm, pore size 0.2 µm) which was pre-soaked in 5xSSC. The filtration was carried out under slight vacuum, the speed was about 1 ml/min.

After filtration the nitrocellulose filters were air-dried and baked in a oven at 80°C for 4 hr. From these filters small pieces of filters (diameter 6 mm, each containing 7-10 µg of Thermoplasma DNA) were made for the hybridization.

6.13.2 Hybridization and RNase digestion

The ^{32}P-labeled 23S and 16S rRNAs (sp. act. ca. 4 µCi/µg) were dissolved at a concentration of about 20 µg/ml in a buffer containing 2xSSC and 25% formamide. These hot rRNA solutions were diluted logarithmically with the same buffer. Hybridization was done by incubating a small filter

containing 7-10 µg of Thermoplasma DNA in 200 µl of each of the serially diluted hot rRNA probes at 60°C for 16 hr.

After hybridization the filters were washed once with 2xSSC and then incubated with 1 ml of a RNase solution (10 µg of RNase A and 50 U of T1) for 1 hr at 37°C. Subsequently the filters were incubated in 1 ml of 2xSSC-25% formamide for 15 min at 60°C, and washed twice with 2xSSC at room temperature. The amounts of rRNA remaining on the filters were estimated by counting the radioactivity of each filter in a liquid scintillation counter (Cerenkov counts) and calculating the radioactivity to the weight. The exact amount of DNA on each of the small filters was determined using the diphenylamine reaction.

7. LITERATURE

(1) Alwine, J. C., Kemp, D. J., Parker, B.A., Reiser, J., Regart, J., Stark, G. R. & Wahl, G. M. (1979) Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. Methods in Enzymol. 68, 220-242.

(2) Bedbrook, J. R., Kolodner, R. & Bogorad, L. (1977) Zea mays chloroplast ribosomal RNA genes are part of a 22,000 base pair inverted repeat. Cell 11, 739-749.

(3) Bell, G. I., Degennaro, L. J., Gelfand, D. H., Bishop, R. J., Valenzuela, P. & Rutter, W. J. (1977) Ribosomal RNA genes of Saccharomyces cerevisiae I. physical map of the repeating unit and location of the regions coding for 5S, 5.8S, 18S and 25S ribosomal RNAs. J. Biol. Chem. 252, 8118-8125.

(4) Bielka, H. & Stahl, J. (1978) Structure and function of eukaryotic ribosomes. in International review of biochemistry, amino acid, and protein biochemistry synthesis II. (Arnstein HRV ed.) vol. 18, pp 79-168. University Park Press, Baltimore.

(5) Birenbaum, M., Schlessinger, D. & Hashimoto, S. (1978) RNase III cleavage of Escherichia coli rRNA precursors fragment release and dependence on salt concentration. Biochemistry 17, 298-307.

(6) Boseley, P. G., Tuyns, A. & Birnsteil, M. L. (1978) Mapping of the Xenopus laevis 5.8S rDNA by restriction and DNA sequencing. Nucleic Acids Res. 5, 1121-1137.

(7) Botchan, P., Reeder, R. H. & Dawid I. B. (1977) Restriction analysis of the nontranscribed spacers of Xenopus laevis ribosomal DNA. Cell 11, 599-607.

(8) Boynton, J., Gillham, N. W. & Lambowitz, A. M. (1980) Biosynthesis of chloroplast and mitochondrial ribosomes. in: Ribosomes: structure, function, and genetics (chambliss et al. eds.) pp. 903-950. University Park Press, Baltimore.

(9) Brosius, J., Plamer, M. L., Kennedy, P. J. & Noller, J. G. (1978) Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 75, 4801-4805.

(10) Brosius, J., Dull, T. J. & Noller H. F. (1980) Complete nucleotide sequence of a 23S ribosomal RNA gene from Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 77,

201-204.

(11) Brownlee, G. G., Sanger, F. & Barrell, B. G. (1968) The sequence of 5S ribosomal ribonucleic acid. J. Mol. Biol. 34, 379-412.

(12) Buongiorno-Nardelli, M., Amaldi, F., Beccari, E. & Junakovic, N. (1977) Size of ribosomal DNA repeating units in Xenopus laevis: Limited individual heterogeneity and extensive population polymorphism. J. Mol. Biol. 110, 105-117.

(13) Chaconas, G. & van de Sande, J, H. (1980) 5'-^{32}P labeling of RNA and DNA restriction fragments. Methods in Enzymol. 65, 75-85

(14) Chow, L. T. & Davidson, N. (1973) Electron microscope mapping of the distribution of ribosomal genes of the Bacillus subtilis chromosome. J. Mol. Biol. 75, 265-279.

(15) Christiansen, c., Freundt, E. A. & Black, F. T. (1975) Genome size and deoxyribonucleic acid base composition of Thermoplasma acidophilium. Int. J. syst. Bacteriol. 25, 99-101.

(16) Cockburn, A. G., Newkirk, M. J. & Firtel, R. A. (1976) Organization of the ribosomal RNA genes of Dictyostelium discoideum: Mapping of the nontranscribed spacer regions. Cell 9, 605-613.

(17) Cory, S. & Adams, J. M. (1977) A very large repeating unit of mouse DNA containing the 18S, 28S and 5.8S rRNA genes. Cell 11, 795-805.

(18) Cramer, J. H., Farrelly, F. W. & Rownd, R. H. (1976) Restriction endonuclease analysis of ribosomal DNA from Saccharomyces cerevisiae. Mol. gen. Genet. 148, 233-241.

(19) Dagert, M. & Ehrlich, S. D. (1979) Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells. Gene 6, 23-28.

(20) Darland, G. & Brock, T. D. (1970) A thermophilic, acidophilic mycoplasma isolated from a coal refuse pile. Science 170, 1416-1418.

(21) De Ley, J. & De Smedt, J. (1975) Improvements of the membrane filter method for DNA:RNA hybridization. Antonie van Leewenhoek, 41, 287-307.

(22) Dennis, P. P. & Nomura, M. C. (1975) Stringent control of the transcriptional activities of ribosomal protein genes in E. coli. Nature (Lond.) 255, 460-465.

(23) De Rosa, M., Gambacorta, A. & Bu'lock, J. D. (1975) Extremely thermophilie acidophilic bacteria convergent with Sulfolobus acidocaldarius. J. gen. Microbiol. 86, 156-164.

(24) de Vries, M., de Jonge, J. C., Bakker, H., Meurs, H. & Kroon, A. (1979) The anatomy of the tRNA-rRNA region of the Neurospora crassa mitochondrial DNA. Nucleic Acids Res. 6, 1791-1803.

(25) Dugaiczyk, A., Boyer, H. W. & Goodman, H. B. (1975) Ligation of Eco RI endonuclease-generated DNA fragments into linear and circular structures. J. Mol. Biol. 96, 171-184.

(26) Dunn, J. J. & Studier, F. W. (1973) T7 early RNAs and Escherichia coli ribosomal DNAs are cut from large precursor RNAs in vivo by Ribonuclease III. Proc. Natl. Acad. Sci. U.S.A. 70, 3296-3300.

(27) Dyer, T. A. & Bowman, C. M. (1979) Nucleotide sequence of chloroplast 5S ribosomal ribonucleic acid in flowering plants. Biochem. J. 183, 595-604.

(28) Engberg, J., Anderson, P., Leick, V. & Collins, J. (1976) Free ribosomal DNA molecules from Tetrahymena pyriformis GL are giant Palindromes. J. Mol. Biol. 104, 455-470.

(29) Erickson, J. M., Rushfold, C. L., Dorney, D. J., Wilson, G. N. & Schmickel, R. D. (1981) Structure and variation of human ribosomal DNA: Molecular analysis of cloned fragments. Gene 16, 1-9.

(30) Falvey, A. K. & Staehelin, T. (1970) Structure and function of mammalian ribosomes II. exchange of ribosomal subunits at various stages of in vitro polypeptide synthesis. J. Mol. Biol. 53, 21-34.

(31) Faye, G., Kujawa, C., Dujon, B., Bolotin-Fukuhara, M., Wolf, K., Fukuhara, H. & Slonimski, P. P. (1975) Localization of the gene coding for the mitochondrial 16S ribosomal RNA using rho mutants of Saccharomyces cerevisiae. J. Mol. Biol. 99, 203-217.

(32) Fox, G. E., Pechmann, K. R. & Woese, C. R. (1977) Comparative cataloging of 16S ribosomal ribonucleic acid: Molecular approach to procaryotic systems. Int. J. system. Bact. 27, 44-57.

(33) Fox, G. E., Stackebrandt, E., Hespell, R. B., Gibson, J., Maniloff, J., Dyer, T. A., Wolfe, R. S., Balch, W. E., Tanner, R. S., Magrum, L. J., Zablen, L. B., Blakemore, R., Gupta, R., Bonen, L., Lewis, B. J., Stahl, D. A., Luehrsen, K. Rl, Chen, K. N. & Woese, C. R. (1980) The phylogeny of prokaryotes. Science 209,

457-463.

(34) Frankel, G., Cockburn, A. F., Kindle, K. L & Firtel, G. A. (1977) Organization of the ribosomal RNA genes of Dictyostelium discoideum, mapping of the transcribed region. J. Mol. Biol. 109, 539-558.

(35) Ghora, B. K. & Apirion, D. (1978) Structural analysis and in vitro processing to p-5 RNA of a 9S RNA molecule isolated from an rne mutant of E. coli. Cell 15, 1055-1066.

(36) Ginsburg, D. & Steitz, J. A. (1975) The 30S ribosomal precursor RNA from Escherichia coli, a primary transcript containing 23S, 16S and 5S sequences. J. Biol. Chem. 250, 5647-5654.

(37) Glover, D. M. & Hogness, D. S. (1977) A novel arrangement of the 18S and 28S sequences in a repeating unit of Drosophila melanogaster rDNA. Cell 10, 167-176.

(38) Goldbach, R. W., Bollen-De Boer, J. E., Van Bruggen, E. F. J. & Borst, P. (1978) Conservation of the sequence and position of the ribosomal RNA genes in Tetrahymena pyriformis mitochondrial DNA. Biochim. Biophys. Acta 521, 189-197.

(39) Goldbach, R. W., Borst, P., Bollen-DeBoer, J. E. & Van Bruggen, E. F. J. (1978) The organization of ribosomal RNA genes in the mitochondrial DNA of Tetrahymena pyriformis strain ST. Biochim. Biophys. Acta 521, 169-186.

(40) Goldberg, M. L., Lifton, R. P., Stark, G. R. & Williams, J. G. (1979) Isolation of the specific RNAs using DNA covalently linked to diazobenzylowymethyl cellulose or paper. Methods in Enzymol. 68, 206-220.

(41) Goldfarb, A. & Daniel, V. (1981) Mapping of transcription units in the bacteriophage T4 tRNA gene cluster. J. Mol. Biol. 146, 393-412.

(42) Green, M. R., Grimm, M. G., Goewert, R. R., Collins, R. A., Cole, H. D., Lambowitz, A. M., Heckman, J. E., Yin, S. & RajBhandary, U. L. (1981) Transcripts and processing patterns for the ribosomal RNA and transfer RNA region of Neurospora crassa mitochondria DNA. J. Biol. Chem. 256, 2027-2034.

(43) Grimm, M. G. & Lambowitz, A. M. (1979) The 32S RNA of Neurospora crassa mitochondria is not a precursor of the mitochondrial ribosomal RNAs J. Mol. Biol. 134, 667-672.

(44) Grivell, L. A. & Groot, G. S. P. (1972) Spinach chloroplast ribosomes active in protein synthesis. FEBS

letters 25, 21-24.

(45) Grunstein, M. & Hogness, D. S. (1975) Colony hybridization: A methos for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. U.S.A. 72, 3961-3965.

(46) Gunningham, R.S., Bonen, L., Doolittle, W. F. & Gray, M. W. (1976) Unique species of 5S, 18S, and 28S ribosomal RNA in wheat mitochondria. FEBS letters 69, 116-122.

(47) Gupta, R. (1982) Transfer RNAs in Halobacterium volcanii and other archaebacteria. Zbl. Bakt. Hyg., I. Abt. Orig. C3, in press.

(48) Hahn, U., Lazarus, C. M., Luensdorf, H. & Kuentzel H. (1979) Split gene for mitochondrial 24S ribosomal RNA of Neurospora crassa. Cell 17, 191-200.

(49) Hartley, M. R. & Ellis, R. J. (1973) Ribonucleic acid synthesis in chloroplasts. Biochem. J. 134, 249-262.

(50) Haseltine, W. A., Lindan, C. P., D'Andrea, A. D. & Johnsrud, L. (1980) The use of DNA fragments of defined sequence for the study of DNA damage and repair. Methods in Enzymol. 65, 235-248.

(51) Hastings, J. R. B. & Kirby, K. S. (1966) The nucleic acids of Drosophila melanogaster. Biochem. J. 100, 532-539.

(52) Heckman, J. E. & RajBhandary, U. L. (1979) Organization of tRNA and rRNA genes in N. crassa mitochondria: Intervening sequence in the large rRNA gene and strand distribution of the RNA genes. Cell 17, 583-595.

(53) Hilpert, R., Winter, J., Hammes, W. & Kandler, O. (1981) The sensitivity of archaebacteria to antibiotics. Zbl. Bakt. hyg., I. Abt. Orig. C2, 11-20.

(54) Hofman, J. E., Lau, R. H. & Doolittle, W. F. (1979) The number, physical organization and transcription of ribosomal RNA cistrons in an archaebacterium: Halobacterium halobium. Nucleic Acids Res. 7, 1321-1333.

(55) Jordan, B. R. & Glover, D. M. (1977) 5.8S and 2S rDNA is located in the "transcribed spacer" region between the 18S and 26S rDNA genes in Drosophila melanogaster. FEBS letters 78, 271-274.

(56) Junakovic, N., Poretti, A., Amaldi, F. & Buongiorno-Nardelli, M. (1978) Differently sized rDNA repeating units of Xenopus laevis are arranged as internally homogeneous clusters along the nucleolar organizer.

Nucleic Acid Res. 1335-1343.

(57) Kandler, O. (1979) Zellwandstrukturen bei Methan-Bakterien. Zur Evolution der Prokaryonten. Naturwissenschaften 66, 95-105.

(58) Karrer, K. M. & Gall, J. G. (1976) The macronuclear sibosomal DNA of Tetrahymena pyriformis is a palindrome. J. Mol. Biol. 104, 421-453.

(59) Kessel, M. & Klink, F. (1980) Archaebacterial elongation factor is ADP-ribosylated by diphthera toxin. Nature (Lond.) 287, 250-251.

(60) Koller, B. & Delius, H. (1980) Vicia faba chloroplast DNA has only one set of ribosomal RNA genes as shown by partial denaturation mapping and R-loop analysis. Mol. gen. Genet. 178, 261-269.

(61) Kramer, R. A., Philippsen, P. & Davis, R. W. (1978) Divergent transcription in the yeast ribosomal RNA coding region as shown by hybridization to separated strands and sequence analysis of cloned DNA. J. Mol. Biol. 123, 405-416.

(62) Langworthy, T. A., Smith, P. F. & Mayberry, W. R. (1972) Lipids of Thermoplasma acidophilum. J. Bacteriol. 112, 1193-1200.

(63) Langworthy, T. A., Mayberry, W. R. & Smith, P. F. (1974) Long-chain glycerol diether and polyol dialkyl glycerol triether lipids of Sulfolobus acidocaldarius. J. Bacteriol. 119, 106-116.

(64) Langworthy, T. A. (1977) Long-chain diglycerol tetraethers from Thermoplasma acidophilum. Biochim. Biophys. Acta 487, 37-50.

(65) Leaver, C. J. & Harmey, M. A. (1976) Higher-plant mitochondrial ribosomes contain a 5S ribosomal ribonucleic acid component. Biochem. J. 157, 275-277.

(66) Loening, U. E. (1967) The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem. J. 102, 251-257.

(67) Leoning, U. E., Jones, K. W. & Birnstiel, M. L. (1969) Properties of ribosomal RNA precursor in Xenopus laevis; Comparison to the precursor in mammals and in plants. J. Mol. Biol. 45, 353-366.

(68) Long, E. O. & David, I. B. (1980) Repeated genes in eukaryotes. Ann. Rev. Biochem. 49, 727-764.

(69) Loughney, K., Lund, E. & Dahlberg, J. E. (1982) tRNA genes are found between the 16S and 23S rRNA genes in

Bacillus subtilis. Nucleic Acids Res. 10, 1607-1624.

(70) Leuhrsen, K. R., Fox, G. E., Kilpatrick, M. W., Walker, R. T., Domdey, H., Krupp, G. & Gross, H. J. (1981) The nucleotide sequence of the 5S rRNA from the archaebacterium Thermoplasma acidophilum. Nucleic Acid Res. 9, 965-970.

(71) Lund, D. & Dahlberg, J. E. (1977) Spacer transfer RNAs in ribosomal RNA transcripts of E. coli: Processing of 30S ribosomal RNA in vitro. Cell 11, 247-262.

(72) Maizels, N. (1976) Dictyostelium 17S, 25S, and 5S rDNAs lie within a 38,000 base pair repeated unit. Cell 9, 431-438.

(73) Malnoe, P. & Rochaix, J. D. (1978) Localization of 4S RNA genes of the chloroplast genome of Chlamydomonas reinhardii. Mol. gen. Genet. 166, 269-275.

(74) Mannella, C. A., Collins, R. A., Green, M. R. & Lambowitz, A. M. (1979) Defective splicing of mitochondrial rRNA in cytochrome-deficient nuclear mutants of Neurospora crassa. Proc. natl. Acad. Sci. U.S.A. 76, 2635-2639.

(75) Manning, R. F., Samols, D. R. & Gage, L. P. (1978) The genes for 18S, 5.8S and 28S ribosomal RNA of Bombyx mori are organized into tandem repeats of uniform length. Gene 4, 153-166.

(76) Matheson, A. T. (1982) Evolution of the ribosomal 'A' protein domain in archaebacteria. Zbl. Bakt. Hyg., I. Abt. Orig. C3, in press.

(77) Mescher, M. F. & Strominger, J. L. (1975) Purification and characterization of a prokaryotic glycoprotein from cell envelope of Halobacterium salinarium. J. Biol. Chem. 251, 2005-2014.

(78) McDonell, M. W., Simon, M. N. & Studier, F. W. (1977) Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophresis in neutral and alkaline gels. J. Mol. Biol. 110, 119-146.

(79) Moran, C. P. J. & Bott, K. F. (1979) Restriction enzyme analysis of Bacillus subtilis ribosomal ribonucleic acid genes. J. Bacteriol. 140, 99-105.

(80) Nomura, M. & Post, L. E. (1980) Organization of ribosomal genes and regulation of their expression in Escherichia coli. in Ribosome: Structure, function and genetics. (Chambliss et al. eds.) University Park Press, Baltimore, pp 761-691.

(81) Orozco, E. M., Gray, P. W. & Hallick, R. B. (1980)

Euglena gracilis ribosomal RNA transcription units, I. The location of transfer RNA, 5S, 16S, and 23S ribosomal RNA genes. J. Biol. Chem. 255, 10991-10996.

(82) Orozco, E. M., Rushlow, K. E., Dodd, J. R. & Hallick, R. B. (1980) Euglena gracilis chloroplast ribosomal RNA transcription units, II. Nucleotide sequence homology between the 16S-23S ribosomal RNA spacer and the 16S ribosomal RNA leader regions. J. Biol. Chem. 255, 10997-11003.

(83) Palmiter, R. D. (1973) Ovalbumin messenger ribonucleic acid translation comparable rates of polypeptide initiation and elongation on ovalbumin and globin messenger ribonucleic acid in a rabbit reticulocyte lysate. J. Biol. Chem. 248, 2095-2106.

(84) Palmiter, R. D. (1974) Magnesium precipitation of ribonucleoprotein complexes, Expedient techniques for the isolation of undetraded polysomes and messenger ribonucleic acid. Biochemistry 13, 3606-3615.

(85) Peacock, A. C. & Dingman, C. W. (1968) Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. Biochemistry 7, 668-674.

(86) Pecher, T. & Boeck, A. (1981) In vivo susceptibity of halophilic and methanogenic organisms to protein synthesis inhibitors. FEMS Microbiol. 10, 295-297.

(87) Pellegrini, M. & Manning, J. (1977) Sequence arrangement of the rDNA of Drosophila melanogaster. Cell 10, 213-224.

(88) Perry, R. P., Chen, T. Y., Freed, J. J., Greenberg, J. R., Kelley, E. E. & Tartof, K. E. (1970) Evolution of the transcription unit of ribosomal RNA. Proc. Natl. Acad. Sci. U.S.A. 65, 609-616.

(89) Perry, R. P. (1976) Processing of RNA. Ann. Rev. Biochem. 45, 605-629.

(90) Perry, R. P., La Torre, J., Kelley, D. E. & Greenberg, J. R. (1972) On the lability of poly(A) sequences during extraction of messenger RNA from polyribosomes. Biochim. Biophys. Acta 262, 220-226.

(91) Pinkerton, T. C., Paddock, G. & Abelson, J. (1973) Nucleotide sequence determination of bacteriophage T4 leucine transfer ribonucleic acid. J. Biol. Chem. 248, 6348-6365.

(92) Potter, S. S., Bott, K. F. & Newbold, J. E. (1977) Two-dimensional restriction analysis of the Bacillus subtilis genome: Gene purification and ribosomal

ribonucleic acid gene organization. J. Bacteriol. 129, 492-500.

(93) Prangishvilli, D., Zillig, W., Gierl, A., Biesert, L. & Holz, I. (1982) DNA-dependent RNA polymerase of Thermoacidophilic archaebacteria. Eur. J. Biochem. 122, 471-477.

(94) Rawson, J. R. Y., Clegg, M. T., Thomas, K., Rinehart, C. & Wood, B. (1981) A restriction map of the ribosomal RNA genes and the short single-copy DNA sequence of the pearl millet chloroplast genome. Gene 16, 11-19.

(95) Rigby, P. W., Dieckmann, M., Rhodes, C. & Berg, P. (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113, 237-251.

(96) Rochaix, J. D. (1978) Restriction endonuclease map of the chloroplast DNA of Chlamydomonas reinhardii. J. Mol. Biol. 126, 597-617.

(97) Rochaix, J. D. & Malnoë, P (1978) Anatomy of the chloroplast ribosomal DNA of Chlamydomonas reinhardii. Cell 15, 661-670.

(98) Ron, LE. Z., Kohler, R. E. & Davis, B. D. (1968) Magnesium ion dependence of free and polysomal ribosomes from Escherichia coli. J. Mol. Biol. 36, 83-89.

(99) Rosen, J. M., Woo, S. L. C., Holder, J. W., Means, A. R. & Malley, B. W. (1975) Preparation and preliminary characterization of purified ovalbumin messenger RNA from the hen oviduct. Biochemistry 14, 69-78.

(100) Sanders, J. P. M., Heyting, D. & Borst, P. (1975) The organization of genes in yeast mitochondrial DNA. I. The genes for large and small ribosomal RNA are far apart. Biochem. Biophys. Res. Commun. 65, 699-707.

(101) Schlessinger, D., Ono, M., Nikolaev, N. & Silengo, L (1974) Accumulation of 30S preribosomal ribonucleic acid in an Escherichia coli mutant treated with chloramphenicol. Biochemistry 13, 4268-4271.

(102) Shine, J. & Dalgarno, L. (1975) Determinant of cistron specificity in bacterial ribosomes. Nature (Lond.) 254, 34-38.

(103) Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

(104) Sturm, S., Schoenefeld, W., Zillig, W., Janekovic, D. & Stetter, K. O. (1980) Structure and function the DNA

dependent RNA polymerase of the archaebacterium Thermoplasma acidophilum. Zbl. Bakt. Hyg., I. Abt. Orig. C1, 12-25.

(105)Sutcliffe, J. G. (1978) pBR322 restriction map derived from the DNA sequence: Accurate DNA size markers up to 4361 nucleotide pairs long. Nucleic Acids Res. 5, 2721-2728.

(106)Tait, A. & Knowles, J. D. C. (1977) Characterization of mitochondrial and cytoplasmic ribosomes from Paramecium cuirelia. J. Cell Biol. 73, 139-148.

(107)Tomioka, N., Shinozaki, K. & Sugiura, M. (1981) Molecular cloning and characterization of ribosomal RNA genes from a green-blue alga, Anacystis nidulans. Mol. gen. Genet. 184, 359-363.

(108)Tornabene, T. G. & Langworthy, T. A. (1979) Diphytanyl and dibiphytanyl glycerol ether lipids of methanogenic archaebacteria. Science 203, 51-53.

(109)Tornabene, T. G., Langworthy, T. A., Holzer, G. & Oro, J. (1979) Squalenes, phytanes and other isoprenoids as major neutral lipids of methanogenic and thermoacidophilic "archaebacteria". J. Mol. Evol. 13, 73-83.

(110)Tu, J., Prangishvilli, D., Huber, H., Wildgruber, G., Zillig, W. & Stetter, K. O. (1982) Taxonomic relations between archaebacteria including 6 novel genera examined by cross hybridization of DNAs and 16S rRNAs. J. Mol. Evol. 18, 109-114.

(111)Valenzuela, P., Bell, G. I., Venegas, A., Sewell, E. T., Masiare, F. R., DeGennaro, L. J., Weinberg, F. & Rutter, W. J. (1977) Ribosomal RNA genes of Saccharomyces cerevisiae, II. Physical map and nucleotide sequence of the 5S ribosomal RNA gene and adjacent intergenic regions. J. Biol. Chem. 252, 8126-8135.

(112)Walker, D. (1982) The nucleotide sequence of the initiator tRNA from Thermoplasma acidophilum. Zbl. Bakt. hyg., I. Abt. Orig. C3, in press.

(113)Wellauer, P. K. & Dawid, I. B. (1977) The structural organization of ribosomal DNA in Drosophila melanogaster. Cell 10, 193-212.

(114)Wellauer, P. K. & Dawid, I. B. (1979) Isolation and sequence organization of human ribosomal DNA. J. Mol. Biol. 128, 289-303.

(115)Wellauer, P. K., Dawid, I. B. & Tartof, K. D. (1978) X and Y chromosomal ribosomal DNA of Drosophila: Comparison of spacers ans insertions. Cell 14, 269-278.

(116)Wellauer, P. K., Reeder, R. H., Dawid, I. B. & Brown, D. D. (1976) The arrangement of length heterogeneity in repeating units of amplified and chromosomal ribosomal DNA from Xenopus laevis. J. Mol. Biol. 105, 487-505.

(117)White, R. L. & Hogness, D. S. (1977) R loop mapping of the 18S and 28S sequences in the long and short repeating units of Drosophila melanogaster rDNA. Cell 10, 177-192.

(118)Wild, M. A. & Gall, J. G. (1979) An intervening sequence in the gene coding for 25S ribosomal RNA of Tetrahymena pigmentosa. Cell 16, 565-573.

(119)Woese, C. R. & Gupta, R. (1981) Are archaebacteria merely derived "prokaryotes"? Nature (Lond.) 289, 95-96.

(120)Woese, C. R., Magrum, L. J. & Fox, G. E. (1978) Archaebacteria. J. Mol. Evol. 11, 245-252.

(121)Wool, I. G. (1979) The structure and function of eukaryotic ribosomes. Ann. Rev. Biochem. 48, 719-754.

(122)Wu, M., Davidson, N., Attardi, G. & Aloni, Y. (1972) Expression of the mitochondrial genome in Hela cells. XIV. The relative positions of the 4S RNA genes and of the ribosomal RNA genes in mitochondrial DNA. J. Mol. Biol. 71, 81-93.

(123)Yang, L. & Haug, A. (1979) Purification and partial characterization of a procaryotic glycoprotein from the plasma membrane of Thermoplasma acidophilum. Biochim. Biophys. Acta 556, 265-277.

(124)Zillig, W., Stetter, K. O. & Janekovic, D. (1979) DNA-dependent RNA polymerase from the archaebacterium Sulfolobus acidocaldarius. Eur. J. Biochem. 96, 597-604.

ABBREVIATIONS

α-32P-dCTP	Deoxycytidine 5'-[α-32P]triphosphate
ATP	Adenosine triphosphate
bis	N,N'-methylene bisacrylamide
bp	Base pair(s)
Ci	Curie(s)
cpm	Count(s) per minute
ct-	Chloroplast, e.g. ct-DNA = chloroplast DNA
d.	Dalton(s)
DBM	Diazobenzyloxymethyl
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
Y-32P-ATP	Adenosine 5'-[Y-32P]triphosphate
Kb	Kilo base pair(s)
M	Molar
min	Minute(s)
mRNA	Messenger RNA
mt-	Mitochondrial, e.g. mt-DNA = mitochondrial DNA
p-rRNA	rRNA precursor
rDNA	Ribosomal DNA which contains the ribosomal transcription unit and the nontranscribed spacer DNA; 16S or 23S rDNA clones, clones which contain at least a part of rDNA transcribing for 16S or 23S rRNA
RNA	Ribonucleic acid

RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
rpm	Revolution(s) per minute
rrn	rRNA operon(s) of E. coli
SDS	Sodium dodecylsulfate
sec	Second(s)
Tris	Tris(hydroxymethyl)-aminomethane
vol	Volume

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